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The cytology of the abscission zone in Mercurialis annua

CECIL YAMPOLSKY

(WITH FIGURES 1–8)

At the height of its vigor in mid-summer, hundreds of insects daily visit the male plants of *Mercurialis annua*. They are attracted by the characteristic odor given off by the many nectaries distributed over the inflorescences and also by the large amount of pollen that is produced. The flowers are short-lived. After dehiscence the anther sacs shrivel up and change from a yellow green color to a deep indigo. Such ephemeral and inconspicuous male flowers produced singly on female plants and which ultimately lead to the production of seed have in the past created the false assumption of parthenogenesis in *Mercurialis annua*.

When plants are hardly more than a month old their sex can be identified and from then on they continue to produce an increasingly large number of flowers. Flower production ceases only when the plant dies. During its life span a male plant produces and then discards tens of thousands of flowers. Whenever a flower is thrown off a truncated pedicel is left behind. The cells of the pedicel remain alive as long as the inflorescence, which is an interrupted spike, remains attached to the plant. When a flower is discarded a scar is left behind and that means a place on the plant through which liquids could escape if no provisions were made to prevent such an occurrence.

In studying the cytology of the male, female and hermaphrodite flowers of *Mercurialis annua* I was impressed with the well defined abscission zones in the region somewhat below the receptacle. R. von Wettstein (1916) found a correlation between the abscission zone in the male flower of *Mercurialis annua* and wind pollination. He was of the opinion that this plant had evolved an adaptive mechanism to further wind pollination. He observed that just before dehiscence, the perianth leaves become recurved so that their tips press against the pedicel. Prior to that the separation of the flower from its subtending structure had started. The separation begins at the periphery of the pedicel and proceeds inward towards the central vascular strand. Curvature of the perianth leaves is brought about by the expansion of specialized cells (*Schwellgewebe*) found at the base of each leaf. The springing back of the free ends of the perianth leaves takes place at the moment when the "ringing" of the flower has reached its maximum. The tips of the perianth leaves press against the pedicel and the pressure so generated lifts the flower and throws it with

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some force into space. As the flower moves through space, the anther sacs split open and the pollen grains escape forming tiny clouds of "dust." In such a manner pollination of the female flowers on female plants is brought about.

My observations on the same plant do not support von Wettstein's contentions for the following reasons:

(1). Functional nectaries are present in large numbers on the male inflorescences.

(2) Many insects visit the male plants attracted by odor and pollen.

(3) Male flowers discharge pollen before falling off.

(4) Male flowers remain attached to the plant after the perianth leaves have been recurved.

(5) Female and hermaphrodite flowers also possess the same kind of abscission zone.

(6) Thousands of male flowers examined showed no extreme reflexing of perianth leaves.

The flowers of *Mercurialis annua* in common with a number of other forms show a precocious fore-shadowing of physiological processes. This advance preparation on the part of the plant to discharge a future function certainly indicates that forces besides physical-chemical relations are at work. The purposefulness implied in von Wettstein's analysis of the process that results in the separation of the male flowers from the parent plant loses significance from the fact that female and hermaphrodite flowers also show the abscission zone long before the flowers themselves function. In such flowers the impetus of fertilization cancels and obliterates all the steps for severing the flowers from the mother plant. Unfertilized female and hermaphrodite flowers do not fall off as do the male flowers through a vigorous sundering of parts. They shrivel, dry up and then fall off; the separation takes place at the abscission layer. Whatever the forces in the plant may be that are responsible for the production of a separation layer, they do not draw sex lines. Actual abscission is apparently dependent upon the particular physiological expression of the flower. When the male flower discharges its pollen, it has come to the end of a physiological process which in turn, and as we shall see later, leads to the immediate fulfillment of the rôle of the abscission zone.

The literature on the abscission layer in plants is extensive. For a comprehensive evaluation of that phenomenon I refer to a work of Pfeiffer (1928) entitled "Die pflanzlichen Trennungsgewebe."

When a flower falls off, a surface is exposed from which substances could readily escape. The safeguards to prevent such loss can be studied and analysed long before the flower drops off. Between the time of the

delimination of the cells concerned in the abscission phenomenon and the complete girdling of the pedicel, a considerable number of cells undergo change in form, function and position. It is apparent that such changes cannot be directly observed as cell motions, but if it were possible to photograph these cells step by step during the time when all the preparations towards the throwing off of the flower go on, one could by projecting the photographs and accelerating the speed of the intervals, secure evidence to show cell movements of a pronounced kind.

In studying the pedicels, cytologically and histologically, in all stages of development I have been able to reconstruct a dynamic picture of the process. The process involves cell differentiation, cell multiplication, the

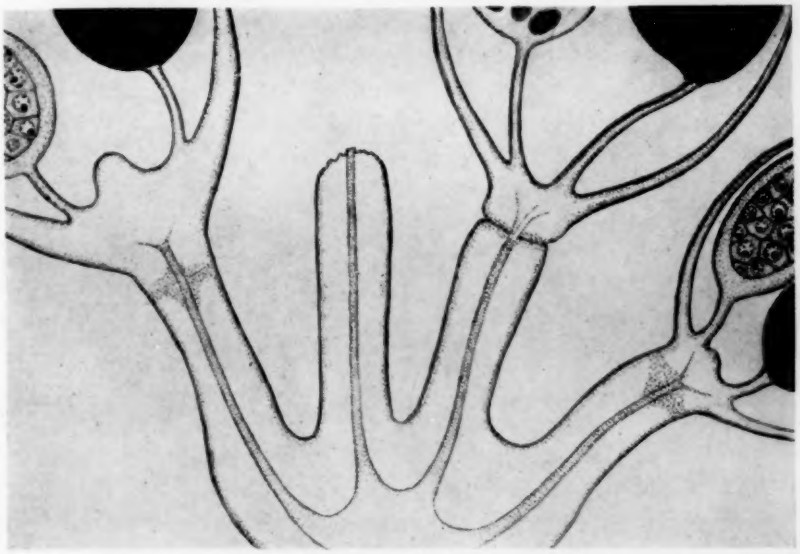

Fig. 1

formation of a definite tissue, cell movements, cell adjustments, the progressive destruction of cells, and finally the synthesis of a fluid which passes out of the pedicel after the flower has fallen off and acts as a plug preventing the escape of liquids.

Figure 1 shows diagrammatically four pedicels in various stages of development. The stippled areas of the two extreme ones show the distribution of the cells that are concerned in abscission. The inner flower to the right shows an advanced stage of the process, the constriction has reached the vascular strand. The fourth pedicel from which the flower has dropped

off shows a ragged end made up of dead cells covered over by the exudation from the stalk itself.

Figure 2 shows a portion of a male flower in the early stages of preparation for abscission. The conspicuously large vacuolated cells are those at the base of the perianth leaf which according to von Wettstein, are concerned with the mechanism of forcibly turning the perianth leaves sharply backward. Immediately below them are the cells of the pedicel

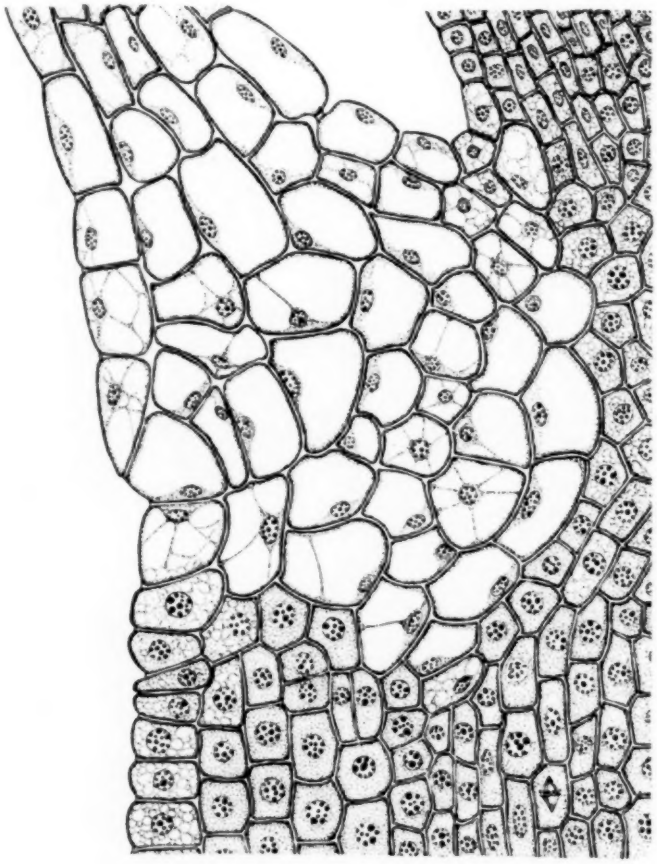

Fig. 2

proper, many of which are meristematic. The two cells of the epidermis that are most compressed show the position of the constriction ring which will move inward like an invisible strangling thread. The other epidermal cells show no form of modification. Next to the epidermal layer division of cells takes place which results in increase in length and circumference of the pedicel as a whole. The meristematic cells have an homogeneous

non-vacuolated cytoplasm in contrast to the non-meristematic cells which are vacuolated. The nuclei of the cells, whether spherical or elongated, exhibit the persistent prochromosomes of *Mercurialis annua*. In a more advanced stage the region above what is to be the abscission layer, grows more rapidly in circumference than that below, giving to the base of the flower a cup-shaped appearance. Large intercellular spaces are present in the perianth leaf tissue and none are present in the pedicel tissue.

Figure 3 shows a portion through a longitudinal cut of a pedicel. It is now wider on top than at the bottom. Cell division goes on actively throughout the whole of that particular region. Fibro-vascular differentiation takes place amidst active nuclear and cell division. The cells to the right and to the left of what is to be the vascular bundle show a striking balance in the rhythm of division. Apparently the impulse that sets the cells of the abscission layer into activity must have travelled radially and must have affected all parts on the circumference at the same time. It is

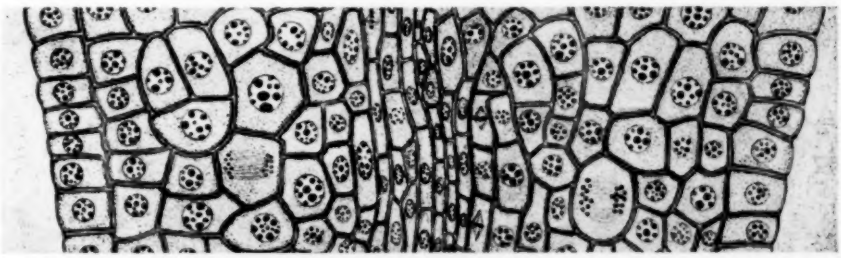

Fig. 3

worth while noting that at this point there is no definite orientation of cells; that occurs later. The two conspicuous cells in the anaphasic stage of nuclear division will of course be divided into two cells whose long axes will be at right angles to what is to be the abscission zone.

Long before the flower opens, preparations for its removal go on. Not only are cells differentiated in the region where the actual constriction will take place but also in the regions below and above. In figure 1 the densely stippled areas of the two outer pedicels show those regions. The value of cytological preparations is apparent when the full length of the pedicel is examined. The contrast between the cells actively concerned with abscission and the rest of the cells, emphasizes how killed and stained material may be an index to profound physiological functions. By a study of these dead cells arrested in their activities a plausible story can be pieced together. The cells concerned in abscission differ from the other cells in size and form and in the appearance of their cytoplasm and nucleus, and

perhaps of even greater significance in the case of *Mercurialis annua*, in the exaggerated size of the prochromosomes that are ever present. Figure 4 shows several of the cells concerned with abscission. The dense and homogeneous cytoplasm is non-vacuolated in contrast to that of the neighboring cells whose cytoplasm is characteristically vacuolated. Such a homogeneous cytoplasm common to all of the cells concerned with abscission speaks for a specialized kind of activity. Equally striking are the prochromosomes and the nucleoli. The appearance of the cytoplasm and

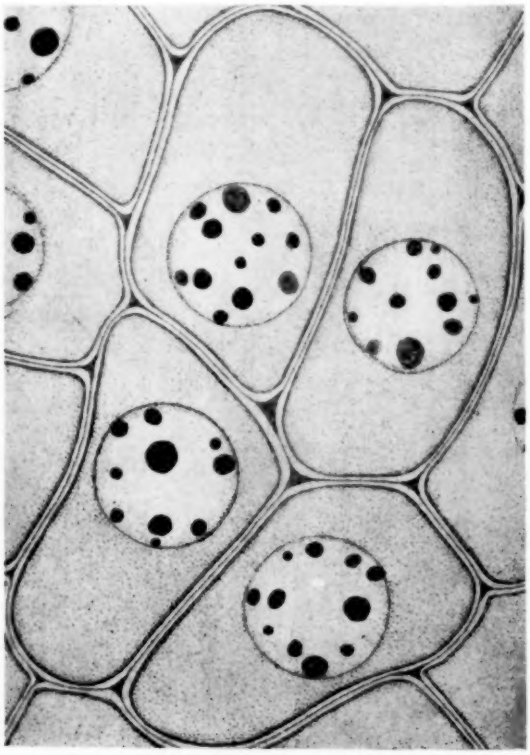

Fig. 4

the nuclei of these cells mark them as especially favorable to the interpretation that the whole cell is actively at work. The prochromosomes are permanent structures. They lie for the most part close to the periphery of the nucleus. They show variability in size. The size of the nuclei and the apparent superiority of the size of the prochromosomes over those of neighboring cells, show that the physiological activities of the cell have a reciprocal influence upon the morphological expression of the parts concerned in carrying out their rôles. Prochromosomes, nucleoli, nuclei and

cytoplasm through their size and texture indicate the tasks that they perform. The four cells indicated in the text figure are representatives of those cells that take part in the abscission phenomena and when a large number are seen at one time, they proclain their function. The nucleolus is the largest of the bodies in each of the nuclei.

When the meristematic cells of the abscission zone have lost the power of further division, a special orientation of the cells becomes apparent. How that is brought about I cannot say. It must be remembered that simultaneously with the activities that are going on in the pedicel, the parts of the flower which it subtends have gone on in their differentiation. The interdependence of parts must not be lost sight of. It must be re-

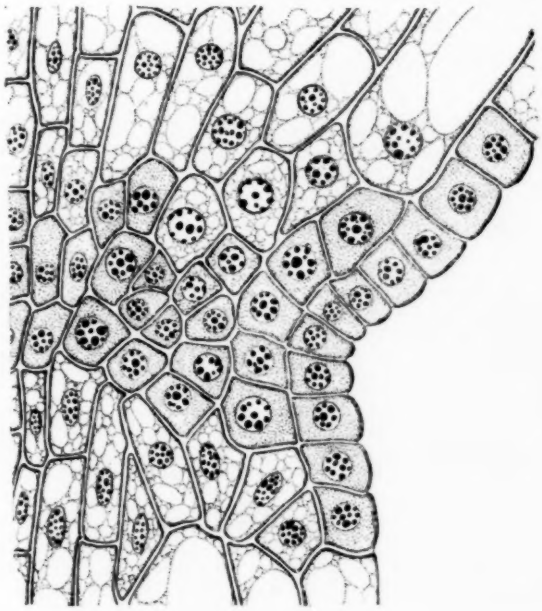

Fig. 5

emphasized that the fore-shadowing of the process of abscission is not an accidental thing. The program included the structures above and below the line of cleavage. It is logical to assume that communication between the parts concerned is always maintained whether it be through movements of substances, through change in pressure relations, through growth hormones, or through impulses travelling from cell to cell. Shifting and readjustment of cells takes place in the abscission zone, especially in that region through which the plane of cleavage is to pass.

Figure 5 shows a portion of what is to be the actual region of cleavage.

Cell division has stopped. The cells with dense and homogenoeus cytoplasm are now more restricted. The casualness of the arrangement of the cells seen in figure 3 has given place to an orderly orientation. Epidermal cells are of two kinds: cubical ones with homogeneous cytoplasm and greatly elongated ones poor in cytoplasm. The intercellular spaces have intreased in size. A dissolution of the cementing substances which had kept che cells rigidly stationary prior to that event must have occurred. The enzymic fluids by digesting the binding material created more room for the closely packed cells. I am convinced from an examination of a very large number of stages that the cells, like individuals released from very cramped positions, stretch and shift and achieve new adjustments in their positions. Harper (1918) has shown in his studies of *Pediastrum* that in the colonial organization of the swarm cells the intercellular spaces are not always uniform, some are larger, others smaller, yet a balance is achieved. The

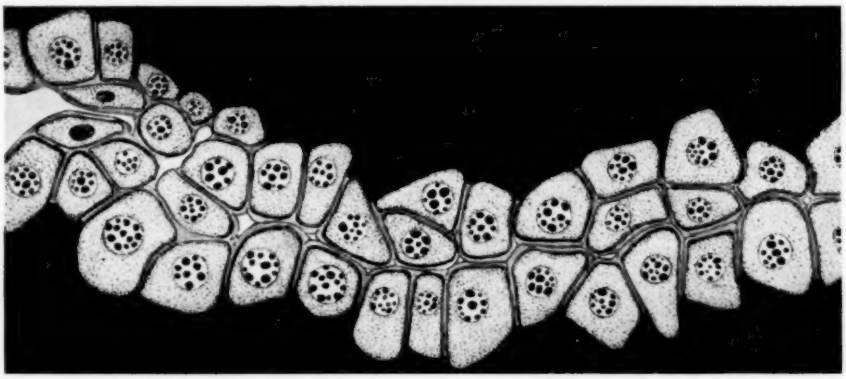

Fig. 6

balance is achieved after a series of trials and errors upon the part of the individual cells until a stable plate is established. In the pedicel of *Mercurialis annua* flowers the cells which are in a more circumscribed environment, hemmed in as they are by other cells, arrive at a stable equilibrium in a manner that is described for *Pediastrum*. As in *Pediastrum* the large number of empty intercellular spaces are not uniform, some are large and others are small.

The actual sundering process starts at the epidermal cells of the pedicel and progresses towards the vascular strand in the manner of an invisible strangling cord. Actual separation is accomplished by two distinct methods: the progressive death of the cells and the destruction of their middle lamellae. Figure 6 shows a portion of the cleavage plane. To the left are two cells that have died. There is no difficulty in recognizing such cells

because the nuclei no longer show differentiation of their contents. All the cells of the layer to which the two above mentioned cells belong have died simultaneously. The cells that are the first to die are the ones with shorter vertical diameters which we saw in figure 2. The middle lamella between the two has disappeared and a gap is formed between them. Intercellular spaces become decidedly more pronounced. Several may coalesce to form a single large one. Death of cells and the production of a cleavage furrow do not necessarily coincide since the cells in the vicinity of the large intercellular spaces have all the appearances of living cells. When the cells next to the dead ones die in turn there is a further coalescence of cavities and great inroads are made upon the circumference of the pedicel leaving the floral structure above in a less firmly held position. The cytoplasm of the cells is still homogeneous but perhaps not as dense. The same brilliancy of staining characteristic of all the cells concerned with abscission, persists in the remaining cells indicating an unabatement of special physiological activity. The orientation of cells shown in figure 5 has reached its climax at the stage shown in text figure 6.

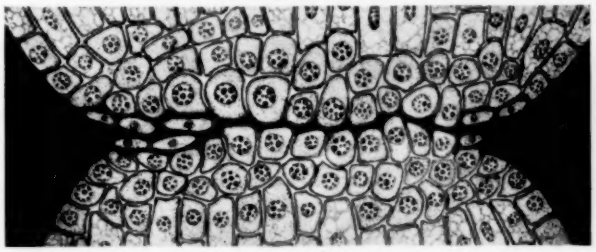

Fig. 7

Figure 7 is a tangential section of the pedicel in a still more advanced stage of cleavage. There are many more dead cells and the split is well on the way towards completion. In this figure the specific orientation of the cells along the cleavage plane is very pronounced. The dissolution of the middle lamellae goes on simultaneously with the dying of the cells. Arrived at the fibro-vascular bundle, the way is barred and at that point the process comes to an end. The final act of cleavage is a mechanical one. By that time the perianth leaves have unfolded and the anthers have dehisced or are about to dehisce. The slender bundle can barely hold the top-heavy burden. The moment comes when the flower snaps off breaking across the conducting cells.

It must not be imagined that the cut is a clean one; we have seen in figure 6 that the cleavage plane, while it tends to remain horizontal, wavers slightly up and down and the result is an irregular surface.

Figure 8 shows a pedicel from which the flower has fallen off. It is composed of dead, dying and living cells. The dead cells are distorted and have cell walls that are greatly swollen. The swelling of the cell walls must have occurred prior to the time the flower fell off since there are no cell contents. To the left and on top, one cell shows a nucleus in the process of disintegration and the cell wall in the process of swelling. The cells below the dead ones are intact and alive. It is interesting to note that the live cells in contact with the dead cells are no longer subjected to reciprocal turgor pressure and their ends have become convexly rounded.

Dead and swelling cells are not sufficiently water-tight to prevent liquids from escaping. Provisions for such an emergency have been made

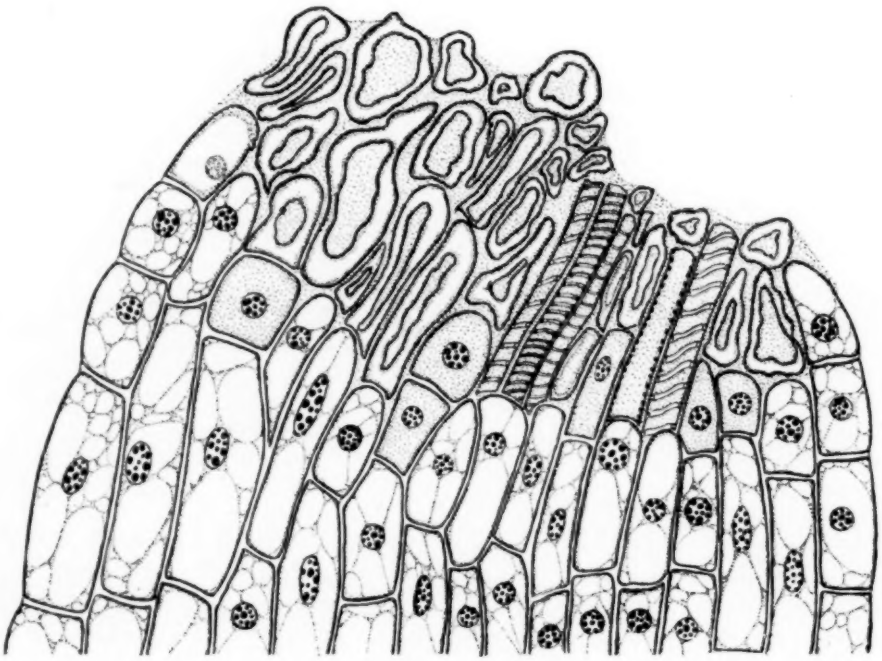

Fig. 8

in the form of a sticky coagulating fluid which effectively plugs up the opening immediately after the break occurs. Here we have a situation analogous to the action of the fibrinogen of the blood. That the substance which escapes from the free end is sticky is evident since pollen grains are often caught and held there. The stippled area surrounding the dead cells indicates the extent of the exudate. Upon coagulation the sticky material shrinks.

In the phenomenon of abscission in *Mercurialis annua* we have the evidence that there is a precocious preparation for future physiological functions. The preliminaries as I have stated before, involve female and hermaphrodite flowers as well as male flowers. Once the process has started it continues but comes to a halt in female and hermaphrodite flowers; it proceeds to its logical conclusion in the male flower when that flower has fulfilled a physiological task, namely dehiscence of the anthers. The process of throwing off a flower involves the production of a large number of cells that take part in the various steps in abscission. It involves also a readjustment, a shifting and realignment of cells. It involves an orderly marshalling of two groups of cells lined up facing one another whose destruction forms a cleavage gap. In the plant of *Mercurialis annua* the casting-off of male flowers is a form of autotomy. It is not, as von Wettstein maintained, an adjustment to insure wind pollination. It is a phenomenon in which a part that no longer functions is discarded. The plant then is more than the sum of its parts; it is an integrated whole. The precocious stages leading to abscission lend strength to that contention.

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Diplocarpon Rosae: from spore germination to haustorium formation

ALICE ARONESCU[1]

(WITH PLATES 16–20 AND ONE TEXT FIGURE)

INTRODUCTION

Previous work on the black spot disease of roses has been concerned with studies on the general morphology and pathology of the fungus causing the disease, and with experiments aimed at the development of suitable methods of control. The purpose of this paper is to present more exact data regarding the conditions favorable for infection, and especially to follow in detail the early stages in the development of the parasite, such as the germination of the spore, the penetration and invasion of the host by the fungus, and the complete development of its vegetative growth. With such facts taken into consideration, it is likely that better methods for control will be worked out, methods which will aim in their application to prevent primary infection as well as the spread of the disease once it has been established.

HISTORY OF THE DISEASE

Fries (1815) describes for the first time the conidial stage and names it *Erysiphe radiosum*. Libert (1826), in France, reports the fungus on *Rosa turbinata*, naming it *Asteroma Rosae* N.

Fries again (1828) describes the fungus under *Erysiphe radiosa* and characterizes it as having a fibrillose radiating mycelium. In 1849 he changes the name to *Actinonema Rosae* (Lib.) reporting the mycelium often sterile and giving a more complete description of the delicate "perithecium" and the two-celled spores. In Rabenhorst's "Deutschlands Kryptogamen-Flora" (1884) the fungus is found under *Asteroma radiosum*. Bonorden (1853) describes it as a new species, *Dicoccum Rosae*, without mentioning any of the earlier reports. Saccardo (1884) accepts *Actinonema Rosae* (Lib.), and reports it on *Rosa centifolia*, *R. gallica*, and *R. rubiginosa*. He gives its distribution as Sweden, France, Brittany, Italy, Belgium, Germany, Austria, Lusitania, and North America.

Trail (1888) in Scotland, describes the fungus on *Rosa tomentosa* as *Marsonia Rosae*, a new species. Independently of Trail's report, Briosi and Cavara (1889) mention the fungus on *Rosa hybrida* under the name *Marsonia Rosae* (Bon.) Br. et Cav. Saccardo (1892) notes that Trail's

[1] Paper presented in substance before the Joint Session of the American Phytopathological Society and the Mycological Society of America. Boston, Mass., December 29, 1933.

Marsonia Rosae is the same as *Marsonia Rosae* (Bon.) Br. et Cav. and that it does not differ from *Actinonema Rosae* previously described.

Scribner (1887a) gives a full description of the disease as it appears in the United States. He reports a series of observations regarding the susceptibility of the leaf in relation to its age and roughness, describes the yellowing and early defoliation caused by the fungus, and realizes the fact that because of defoliation the plant is forced to put out new leaves continuously, a condition which brings it to an exhausted state for the following year. He reports his observations on climatic conditions favorable for infection, on the spreading of the mycelium through the leaf, browning of the leaves, overwintering and control.

Humphrey (1889) pointed out that the perfect stage of the fungus is probably saprophytic on the overwintering leaves. Maynard (1889) undertook experiments on controlling the disease. He found the evaporated sulphur method successful in the greenhouse. In 1895 McAlpine noted the occurrence of the disease in Australia. Halsted (1902) reported having observed the disease in 1892 on *Rosa humilis*, a wild species.

The first detailed scientific study of the organism is made by Ducomet (1907). After a brief statement regarding susceptibility and a description of the symptoms and the effects of the disease, he pays more attention to the later stages of the invasion of the host by the fungus. He illustrates the manner of association of subcuticular hyphae to form the system of strands characteristic of the fungus. Good descriptions and illustrations of the formation of acervuli are also given. Ducomet pictures the fungus as sending "intracellular hyphae" into the host cells and reports the absence of haustoria.

Wolf (1912a, 1912b, 1913) discovered the perfect stage of the parasite and proposed the name *Diplocarpon Rosae* Wolf. Through artificial inoculations starting with ascospores he connected both stages of the fungus. The importance of the ascosporic stage in carrying the fungus over winter is stressed. He also noted that the ascospores do not germinate unless in contact with the rose leaf.

Chifflot (1914) observed that the petioles, stipules and the stem at the base of the diseased leaves may also show infection at times and that even sepals and petals are attacked, a fact confirmed later by Green (1931). Chifflot is of the opinion that the fungus can penetrate quite deeply into the tissue of the stem and infect the young axillary buds which could spread the disease when grafted.

The seriousness of the disease in greenhouses during winter and its relation to temperature and moisture was brought out by Massey (1917).

He determined also (1918a) that the amount of defoliation varied directly with the percentage of infected leaves.

Alcock (1918) and, more recently, Green (1931) mention the fact that the perfect stage of *Actinonema Rosae* cannot be found in Great Britain. They also give a good account of the varieties that show infection on young stems. According to Alcock the spore-bearing acervuli in the young shoots are of two kinds; some develop immediately beneath the cuticle and others are formed in deep cortical cavities. Both authors state that the spores are not found in two-year old acervuli. Sections of young infected shoots of the very susceptible variety Juliet analyzed in January and February (Green, 1931) revealed acervuli full of spores which germinated in water at 24°C.

Other papers on black spot have appeared from time to time (Massey and Westcott, 1928; Massey and Parsons, 1931; Dodge, 1929, 1932; Howitt, 1930; White, 1930a, 1930b, 1932; and Green, 1931). These authors discuss particularly susceptibility and climatic conditions in connection with infection and they also give results of their experiments on different methods of control.

In a comparative study of the strawberry leaf scorch fungus, *Diplocarpon earliana*, and the black spot fungus *Diplocarpon Rosae*, Wolf (1926) corrects certain statements previously made, and also adds some interesting facts. He states, in this paper, that the hyphae of the parasite are intercellular and that "oval to elliptical haustoria penetrate the host cells." He describes, the first time for this fungus, spermatia that may be found in spermogonia at any time during the period from October until early in January. The spermatia may arise in separate acervuli similar to those which bear conidia, or they may be formed in the same acervulus with the conidia.

Dodge (1931) reports that inoculation experiments show that even Red Radiance may be readily infected in the greenhouse. Spotted leaves which had overwintered in wire cages, were inspected by him two years in succession without his finding any ascocarps. He shows conclusively that the mycelium is intercellular and not intracellular, and that numerous rather long haustoria are present especially in the epidermal cells.

SYMPTOMS OF THE DISEASE AND LIFE CYCLE OF THE FUNGUS

The disease can be recognized from the beginning of its development by dark spots with fringed borders about one centimeter in diameter which appear mostly on the upper surface of the leaves. Pale brown spots, with a few acervuli, can sometimes be seen on the lower surface of infected

leaves. The irregular border of the spot is due to the ramifying strands of parallel hyphae which can be seen in young spots radiating from the points of infection.

Alcock (1918), Massey and Westcott (1928), and Green (1931), give detailed lists of Hybrid Teas, Climbers, Hybrid Perpetuals and soft-wooded varieties with stem infections. The variety Los Angeles is more subject to stem infection than any other rose in the New York Botanical Garden. White (1930a) reports also heavy stem infections on this rose and also on Etoile de Feu.

Massey (1918a) and White (1932) show that defoliation is directly correlated with the amount of infection. The yellowing may be confined to the region immediately surrounding the spot, or it can extend much beyond the tissue actually invaded. Yellowing and defoliation seem to vary with the different varieties, with the intensity of infection in the same variety, and with the number of spots on the leaf. In the case of Golden Ophelia the chlorotic condition appears soon after infection and defoliation is abundant. Red Radiance, on the other hand, does not show much yellowing of the leaves, which may remain on the branches a long time after the disease has manifested itself. Grieve (1930) is of the opinion that because of the defoliation the plant becomes starved, and a "die-back" condition begins.

Acervuli are found on the affected parts a few days after the appearance of the spots. They occur mostly on the upper surface of the leaves and only rarely, and a long time after the infection, are they found on the lower surface, in which case a brown spot is evident. They are covered by the cuticle which ruptures irregularly.

At about the end of October and until about January, spermatia are formed (Wolf, 1926) either together with the conidia, filling up the upper part of the acervulus, or in separate fruiting bodies. Late in the autumn, following defoliation, the mycelium invades the deeper layers of the dead leaf and forms a stroma between the epidermis and the palisade parenchyma. Wolf says that archicarps are formed in great abundance during October, and were noted by him also in November and December. Apparently, if all conditions for fertilization (if such is necessary) and for the development of ascocarps are provided, apothecia with asci may be expected in the spring.

The apothecia open by rupturing. The ascospores are not projected violently from the asci; they merely pile up in a whitish heap in the opened ascocarp. Apparently they are spread by wind and rain. If unfavorable conditions for formation of asci are present (Dodge, 1931), these deep-seated fruiting bodies are filled with apothecial conidia cut off in the early

spring from the upward growing filaments. In the spring of 1933, the writer found but very few ascocarps either on the decaying leaves from the rose garden, or on artificially inoculated leaves that had overwintered in wire cages. Many subepidermal acervuli with apothecial conidia, however, were found on almost every infected leaf. Scribner (1887a) is of the opinion that the new shoots in the spring are infected by the spores lodged upon buds at the bases of the petioles, by the water trickling down the leaf-stalk. Frank (1896) says that the mycelium can develop saprophytically from new acervuli which can overwinter in that condition, and discharge the spores in the spring. Wolf (1912b) could not find, in the winter time, acervuli which were bearing conidia. Green (1931) states that on one year old stems acervuli are formed in large numbers in the autumn but they remain dormant and unbroken during the winter months. We have found in the spring on the same leaves with the subepidermal acervuli, the subcuticular acervuli with spores. Overwintering of the parasite and new infections do not depend, therefore, entirely on ascospores. The apothecial conidia formed in the subepidermal acervuli on the decayed leaves, as well as the conidia from deep-seated and superficial acervuli on infected canes, (Alcock, 1918), can start the new infection in the spring and complete the life cycle of the fungus.

ARTIFICIAL INOCULATIONS

Frank (1896) tried artificial inoculations with the conidia and reported having obtained good spots on the tenth day.

Infections from ascospores were obtained by Wolf (1912b). He observes that germination of these spores takes place in 24 hours, but only on the leaf. The inoculated plants which were kept under bell jars for two days, showed small black areas after ten days. Seventeen days from the time of inoculation, mature acervuli appeared. Inoculations made by the same author in petri dishes lined with moist filter paper, showed the radiating strands within four days. Massey and Westcott (1928) succeeded in obtaining good conidial infections in the greenhouse at about 75°F. The experiments undertaken by Dodge (1931) were mostly for the purpose of seeing whether the nature of its cuticle makes Red Radiance a more resistant variety.

Materials and methods

The writer used potted plants which were stimulated to make vigorous growth by adding fertilizer a few weeks before the experiments were started. The varieties chosen were mostly Radiance and Red Radiance, said to be very resistant, and Felicity, Charles K. Douglas, Mrs. F. R.

Pierson, Henry Ford, and Flammenrose, more susceptible types. Young leaves were usually selected for infection, although older leaves were also inoculated when comparative results were desired. From two to six rings were marked on each leaf blade with India ink. With the aid of a small glass rod, drops of a suspension of spores obtained from acervuli or from aritficial cultures, were placed inside of each ring. With a little experience the drops can be made to adhere very quickly to the surface of the leaf so that it is quite improbable that the leaves become injured. In the case of the young leaves of Red Radiance it was somewhat more difficult to make the drops adhere. Suspensions of different densities were used without much difference in the general results. The inoculated plants were placed under the "iceless refrigerator" described by Hunt (1919) which provides a fairly saturated atmosphere for the plants. Thus, in most cases, the drops originally placed on the leaves could still be found even three days afterwards when the plants were usually taken out and placed on the benches. The temperature in the inoculation chamber was always from about eight to twelve degrees lower than it was in the greenhouse.

Humidity and temperature

The first inoculation experiments were started merely for the purpose of finding out whether the fungus is heterothallic. It soon became apparent, however, that temperature and humidity were more responsible for the varying results than were the varieties used. In this experiment a large number of leaves of different ages and varieties were inoculated and observations were made in order to determine the length of time required for the first sign of infection to appear. Countings were made for the number of spots in general, for the number of affected leaves according to their age and also according to the variety. About 2200 leaves were individually inoculated in November 1932, in 30 series. An additional 1000 leaves were inoculated in September 1933, in 11 series. Infection spots were noted in all series between the fourth and the sixth day after inoculation.

No important differences were observed when Red Radiance, Felicity, Radiance, Charles K. Douglas and Mrs. F. R. Pierson were inoculated. In the case of Flammenrose and Henry Ford, infections usually appeared from one to two days later. Furthermore, the spots did not seem to increase in size as quickly and remained more restricted in area.

In general, about 92% of the inoculations were successful. Younger leaves showed practically 100% infection. In a few cases infections were obtained on leaves that had ceased growing before inoculation. The spots however, remained very small and no acervuli, or only very few, were formed.

A second set of experiments was started to determine, as far as our greenhouse conditions permitted, the relation between humidity and temperature and the rapidity of infection. For this reason inoculations were made on a smaller number of leaves at convenient rainy periods. Weather conditions two or three days prior to the inoculation were taken into consideration. Inoculations, for example, made on July 12, August 7, and September 25, gave about the same results. The temperature in the greenhouse during these three experiments was, in general, not higher than 92°F. and not lower than 72°F. Each inoculation was preceded by two days of rain or at least partly rainy and partly cloudy weather. Practically 100 % of infection was recorded and good spots showed in most cases the third day after inoculation or on the fourth day, at the latest. In plate 16 is shown a plant of Red Radiance where all the inoculated places showed good evidence of infection on the third day and the spots had developed beautifully by the second week.

Plants in another group were inoculated on July 12, 1933. No rain was registered for the six preceding days and the temperature varied between 89°F. and 102°F. Infection was not visible until the sixth day. In a set of inoculations started on October 5, when the temperature was about 50°F. in the greenhouse, infection was much retarded, and spots appeared variously from the fifth to the eighth day.

Another series of experiments was conducted to determine whether it is sufficient merely to provide a humid atmosphere or whether the spores must actually be in a film of water. One plant was inoculated on July 10, and was left on the greenhouse bench so that the drops dried out immediately. The atmosphere was quite dry, no rain being recorded during the previous three days. No infection occurred. On August 21, after two rainy days, the same experiment was repeated with four plants. One plant was left out in the greenhouse bench so that the drops dried out about an hour after inoculation; another was left on the bench until after the drops dried out, and then it was placed in the inoculation chamber; a third was left for about 20 hours in the damp chamber and then taken out; the fourth plant was kept in the damp chamber three days as usual. In the case of the first plant where the spores must have germinated in the humid atmosphere of the greenhouse, the spots appeared on the fifth and sixth days. The other three plants showed good infections on the third day. It is apparent from these series of experiments that humid atmospheric conditions, under favorable temperature, are sufficient to provide germination of spores and infection.

Under favorable conditions, large numbers of acervuli appear about nine days after the inoculation is made. The mycelium advances gradually

into the lower tissue of the leaf, so that a brown spot can be seen on this side about two weeks later, but acervuli, if they appear here are developed about a month later and in small numbers.

Good infections, under the same conditions as cited above, were obtained by placing the inoculum on the lower side of the leaf. The percentage of infection is a little lower, due probably to the more unfavorable position of the spores in the drop of water. When infection does follow, a light brown spot is seen on the third or fourth day after inoculation. The mycelium progresses rapidly towards the upper side of the leaf where a distinct but more irregular spot develops and acervuli appear as early as the eleventh day after inoculation, while on the lower side, where the inoculum was actually placed, they did not form until after about a month. These results appear very interesting from the point of view of physiological conditions required by the fungus to develop in the leaf. Perhaps it is the larger quantity of assimilated substances on the upper side of the leaf that makes the fungus spread and develop acervuli first in this region.

Inoculations were also made on the petioles and the stems. A large number of infected petioles were obtained. Under favorable conditions the spots appeared after about the sixth day. Later on, acervuli with large quantities of spores develop. Inoculations on the stem were not so successful. Only very young stems showed infection on about the ninth day and no difference in susceptibility could be found in the varieties that were used.

Artificial inoculations were tried also in the field. On July 14 at 4 P.M, eighty leaves from eleven different varieties were individually inoculated. the places being marked with India ink as usual. It was a warm clear day. The temperature reached above 80°F. and no rain had been recorded for the preceding eight days.The drops of water dried out almost immediately. The next day was cloudy and rain followed on the second day after the inoculation. Ten days after the inoculation, ten leaves distributed among five varieties showed infection. Another series of infections was made on twenty-five leaves of Red Radiance on September 9 at 12 P.M. A heavy rain, which lasted for about two hours, followed very soon after the inoculation. The next few days were fair or partly cloudy. The fourth day spots appeared on sixteen leaves showing that conditions for infection in the field are not much different from those required for infection in the greenhouse. Even after a long rain which followed very soon after the inoculation, enough spores were left on the leaf to produce a good infection. From the preparations of infected material made in order to follow penetration, we observed that even if the drop had not had a chance to dry before the leaves were boiled, many spores still stuck to the surface of the leaf.

Light

Other experiments were made to determine whether plants kept in total darkness could be infected. One inoculation chamber was placed in a photographic dark room, and another one, for controls, was placed in the light, close by the dark room. The temperature registered by minimum and maximum thermometers placed very near the plants showed only very slight differences in the two chambers.

Three series of experiments were made. In the first two series the plants were removed from the dark room three days after inoculation and taken with the controls back to the greenhouse. Infection appeared on the fifth day on the controls and on the sixth day on the plants kept in the dark. The amount of infection was a little higher for the controls. These small differences were not considered important, because they may also occur among different plants inoculated under the same greenhouse conditions. In the third experiment the plants were left seven days in the dark room. No defoliation occurred and no marked chlorotic condition was observed. The plants showed good spots and acervuli developed after the usual interval of time.

Another experiment was made in order to observe whether infection would appear earlier if only the infected leaves were kept in the dark while the rest of the plant was exposed to light. For this purpose the inoculated leaves (including the petioles) were covered with tinfoil. Good spots appeared first on the fourth, and others on the fifth and sixth days, while the controls in the inoculation chamber showed infection, in general, either on the fourth or the fifth day.

Fromme (1913) has shown that total light exclusion either early or late in the incubation period of *Puccinia coronifera* on oats, completely checks the growth of the fungus for the length of time the host was kept in the dark. He explains this retardation as due mostly to the lack of assimilated food and not to the total inability of the fungus to develop for a short time in the dark. In our case it seems that the fungus can develop very well in the dark and that even the reduced supply of food in the leaf is still sufficient for the parasite.

The inoculation experiments, in general, suggest that the best conditions for infection are a saturated atmosphere and a medium temperature of about 75°–80°F. In these cases, spots appear as early as three days after inoculation. Under these conditions, as will be noted later, infection is assured if the spore is provided with a humid environment merely for the first fifteen hours.

INFECTION IN NATURE, RESISTANT VARIETIES, IMMUNITY

All those who have worked on black spot control and have kept records on the degree of infection of the same variety through a few years, agree that there is no completely immune variety. They also agree that immunity and seriousness of infection depend to some extent on climatic conditions.

Numerous and often contradictory records on susceptible varieties are found in the literature. Scribner (1887a) and Frank (1896) report that climbing roses and moss roses and those with rough leaves and hairy and thorny stems seem to suffer more than other kinds. According to Ducomet (1907) *Rosa gallica*, *R. centifolia*, *R. rubiginosa* and *R. indica* exhibit a marked degree of resistance. Chifflot (1914) states that all kinds of roses can be attacked. Green (1931) mentions in passing, that the thin leaved varieties are the most susceptible and thus are the first attacked. The roses with thicker and tougher leaves resist infection better up to the middle of the season when they may become more or less spotted.

Massey (1918a) is the first one to make careful observations on resistant varieties from his extensive experiments on black spot control undertaken at Cornell University and also at different nurseries. In 1928 he considered the Hybrid Perpetuals and the Pernetiana group the most susceptible. Later, however, Massey and Westcott (1928) place the Austrian Briers and the Polyanthas at the head of the list.

Dodge (1932) reaches the conclusion that probably no variety is really immune to black spot. From our own observations of plants in the same rose garden made during the summer of 1933, one should not place too much confidence in reported "resistance." There are certainly different degrees of resistance among the Climbers, however. Star of Persia, a yellow rose, was very badly diseased while other varieties like Paul Noel, Ben Stad, Mary Lovett and Daybreak which grew near it, remained free from infection. After a period of a few successive and quite long rainfalls even Red Radiance showed a number of spotted leaves. One might question whether the little resistance shown here is due to internal conditions in the plant or whether the infection is usually impossible because the waxy substance on the surface of the cuticle normally prevents drops of water from adhering to the young leaves. The writer has always found it more difficult, in making the artificial inoculations in the greenhouse, to make the drops of spore suspension adhere to young leaves of this variety.

Some varieties may escape infection as pointed out by Massey and Westcott (1928), and "although there is doubtless evidence that varieties differ in degree of susceptiblity, it is difficult to classify on this criterion,

based only upon observations, because the factor of klendusity varies with the season, locality, and even the location in the garden."

PENETRATION, INVASION AND HAUSTORIA

Review of the literature

In the case of the fungi that penetrate the unwounded tissue of the leaf instead of entering through the stomatal openings, a much more complicated process must be explained. There are many factors which work together and make it possible for the fungus to penetrate through the outer wall of the epidermis.

There seems to be a general agreement that in order that penetration may be possible, an intimate contact between the hyphae and the host is necessary. This is better accomplished, in most cases, with the aid of an appressorium. The first one to observe the function of these organs was Frank, who named them "Haftorgane" (1883), and showed that they were not "secondary spores" as considered by Fisch (1882), Scribner (1887b) Halsted (1892) and others. De Bary (1886) in his study of the disease caused by different species of *Sclerotinia* is of the opinion that two conditions are necessary for the formation of these organs. There must be a mechanical stimulus produced by the contact between the hypha and some hard substance such as glass of the culture chamber, or the cuticle of the host, and that the fungus must grow in a film of water and not in a nutrient medium. If the spores are germinated in nutrient solutions, penetration occurs also, but no appressoria are formed. His explanation for this is that the solution favors the formation of a larger amount of toxic substances which permit penetration immediately after the hypha has touched the epidermis.

Ward (1888) showed that the tips of the hyphae of *Botrytis cinerea* when coming in contact with the cover slip flatten out and form appressoria. Büsgen (1893) in the case of *Fusicladium pyrinum*, Miyoshi (1895) and Hasselbring (1906), are in this respect, of the same opinion as de Bary, stating that the contact between parasite and host has the effect of forming appressoria and that nutrient solutions used for the germination of the spores suppress their appearance. Büsgen (1893) and later Blackman and Welsford (1916) observed that appressoria, besides making an intimate contact between host and fungus, probably serve as centers of support during the act of penetration.

Numerous cases have also been found where appressoria were not formed and their absence could not be explained by either of the interpretations proposed above. Boyle (1921) finds appressoria of *Sclerotinia Sclerotiorum* formed sometimes where there are good nourishing condi-

tions. Büsgen (1893), Pole Evans (1907) and Waterhouse (1921) working with different rusts, Blackman and Welsford (1916) with *Botrytis cinerea*, Weber (1922) and Bensaude and Keitt (1928) with other fungi, report in certain cases little or no formation of appressoria under conditions similar to those reported by others to be favorable. All these cases might indicate that it is sufficient to have contact between hypha and host without the presence of appressoria.

We may now consider what were the opinions of different authors as to the means by which the little infection peg penetrates the cuticle and the other layers of the cell wall.

De Bary (1886) after making artificial inoculations with *Peziza Sclerotiorum* on different plants, came to the conclusion that penetration is accomplished mostly through chemical means. The substance that makes penetration possible is, according to him, secreted by the fungus. This substance contains a series of organic acids, mostly oxalic, which kill the protoplasts of the cells underneath the drop of spore suspension. It contains also an enzyme which has the power to soften and change the chemical composition of the cell walls after the living contents of the tissues have been killed and, in this manner, open the way for the penetrating hypha. Ward (1888) in the study of a lily disease caused by *Botrytis cinerea* and Büsgen (1893) in a series of observations on Hyphomycetes, Erysiphaceae and Uredineae agree somewhat with de Bary. These authors do not insist, however, on the presence of an organic acid which would first kill the cells. Nordhausen (1899) in studies on mosses and higher plants, is of the opinion that the entrance of the penetrating hypha is possible after the decomposition of the cell wall by a ferment which attacks the celluose. The fungus nourishes itself from the products of this chemical process and then secretes another kind of substance which kills the contents of cells that had previously offered resistance. He could not find any oxalic acid present.

Smith (1902) in his paper on "The Parasitism of *Botrytis cinerea*" is of the same opinion as de Bary, namely, that the killing of the cells takes place first. However, he considers the cellulose enzymes to be subordinate in effect to some toxic substance of a different nature, a soluble substance that diffuses through the cuticle and cell walls and kills the cells ahead of the fungus filaments. He has no doubt that this substance is oxalic acid which almost invariably accompanies the growth of *Botrytis cinerea* and is formed in the metabolism of carbohydrates.

Miyoshi (1895) and Gibson (1904) offer a much more complicated explanation for the entire process. From experiments with *Botrytis cinerea* and *Penicillium glaucum* on cellulose and collodion membranes and also on onion scale epidermis, Miyoshi concludes that the mechanical force

exercised by the fungus as it grows, accompanied by the action of some softening enzymes, could make penetration possible. It is of utmost importance, though, that these be preceded by a chemical stimulus which emanates from the host plant. This stimulus is of great significance because it induces the formation of powerful appressoria and infection threads. Gibson (1904) thinks that the chemotropic substance is not a special one because some rusts show good penetration in plants which are not their hosts. The penetrating hyphae die, of course, in these cases before formation of haustoria. The influence of the exosmosis of nutrient substances from the host tissue into the infection drop was partly confirmed in the more recent studies by Brown (1922) and Dey (1933).

As we can see from the above, no difference was made, in explaining penetration, between the chemical composition of the cuticle and the rest of the outer wall of the epidermal cell.

Von Mohl (1847) and de Bary (1887, p. 75) have already considered the outer wall of the epidermis to be formed of different layers among which the top one is the cuticle made of cutin, the intermediate ones are layers of cellulose which are permeated by cutin, and were called "Cuticularschichte" by the first author. The innermost are layers of pure cellulose. Recent studies in the constitution and chemical composition of the cell wall undertaken by Lee and Priestley (1924), Lee (1925), Anderson (1928, 1934) and others, have shown that cutin is a complex mixture of fatty substances consisting mainly of free fatty acids and soaps. Since no glycerol has been identified it would seem that the fatty deposit has more of the characteristics of a wax, rather than of a fat. It is, therefore, more probable that the cuticle could be penetrated more easily by mechanical means and that the enzymes which produce cellulose decomposition could not have the same effect on cutin. In the light of these researches on the composition of the cell wall a series of very elaborate studies on the physiology of parasitism undertaken at the Imperial College of Science and Technology has led to a better understanding of this whole subject of penetration.

Brown (1915, 1916), for example, has shown that the extract from young hyphae of *Botrytis* has a double action: a, solution of certain constituents of the cell wall, resulting in loss of coherence of the tissue, and b, death of the protoplasts. He has evidence that both the lethal and the macerating actions are due to the same substance, an enzyme. The cuticle presents in all cases an impenetrable obstacle to the diffusion of the extract. Microscopical studies carried on by Blackman and Welsford (1916) on *Botrytis cinerea*, Boyle (1921) on *Sclerotinia Libertiana*, and Waterhouse (1921) with infections of *Berberis vulgaris* by sporidia of *Puccinia graminis* are in ac-

cord with Brown's results. They come to the conclusion that the piercing of the cuticle is due solely to mechanical pressure exerted by the germ tube as a whole or by a special outgrowth from it. It is made easier by the presence of the mucilage which holds the germ tube firmly in position and enables it to exert the appropriate pressure. As soon as the cuticular barrier is passed, enzyme action can occur as is shown by the swelling of the subcuticular layers.

With this discussion in mind, we may now consider the manner by which *Diplocarpon Rosae* penetrates the rose leaf.

Studies on penetration and invasion of the host

Even though one may make many cytological preparations from embedded material, the difficulty of finding a sufficient number of cases showing actual penetration clearly is so great that one is forced to try other methods for this phase of the study. The most satisfactory method for a study of the early stages of penetration was found to be the examination, from above, of the inoculated portion of the leaf under oil immersion. The following method gave the best results.

Drops of spore suspension were placed on the leaves as described previously and, after different intervals of time, leaves were collected and boiled in 95% alcohol for about fifteen minutes to remove the chlorophyll. They were then left in 70% alcohol to regain their softness. The part of the leaf covered by the drop of suspension was cut out and stained in cotton blue dissolved in lactophenol. The fungus only takes the blue stain, while leaf tissues remain unstained. A few hours in the stain is sufficient for showing the early stages of penetration. For later stages, where parallel hyphae are already present, better results are obtained if the pieces are left in the stain for 48 or even 72 hours, because diffusion of cotton blue takes place only through cracked cuticle and through the cut edges of the leaf. The pieces of leaf were mounted in lactophenol, under a thin cover glass. The diffusion of the stain continues slowly so that in a few days the details may be observed much more clearly. The great advantage of this method over the embedding method is that it takes very little time to prepare a number of slides, and each slide prepared shows many stages of penetration which can be observed in detail and in all phases by bringing different depths of the cell into focus. In studying certain cytological details of the formation of haustoria and also of old stages of penetration described in this series, the author has used sections stained with Flemming's triple stain.

The figures, plates 17–20, present a series of drawings made mostly from cotton blue preparations. Our intention in shading the drawings was to show different depths in the cell with the parts of the fungus invading it.

Darker shading represents greater depth in the cell. The contents of the spores and their superficial germ tubes are purposely omitted so as to make it plain that everything not shaded is on the surface of the cuticle. The drawings were made at different magnifications as the case required but the fungus and the host cells were drawn somewhat in their natural proportions. For convenience, proportions were not respected in the text figure. The width of the vertical wall of the host cells varies considerably and appears wider under the microscope at the very top of the cell than it does if you focus a little lower down into the cells.

It was observed that even after boiling and handling the inoculated leaves, many non-germinated spores would also stick to the leaf surface, indicating again that there must be some mucilaginous substance surrounding the spores when they ooze out of the acervulus.

Under conditions favorable for infection, germination is completed within about nine hours after inoculation. Approximately 80% of the spores present on the leaf germinate. Commonly only one of the cells of the spore germinates, and more frequently it is the larger one. Germination may occur at any place in the cell wall but generally appears either at the tip of the cell (pl. 17, fig. 1, a) or at the middle, near the separating wall (pl. 17, fig. 1, b). The cell that is ready to germinate swells and the spot where germination will take place shows a deeply stained shield around it (pl. 17, fig. 1). Sometimes it looks as if the cell were going to germinate in three places (pl. 17, fig. 1, c).

At these spots, now marked by the stain, the spore sends out a germ tube which varies in shape and size. Sometimes, as shown in figure 2, the germ tube is only a short neck-like outgrowth which swells up immediately into an appressorium. Figure 3 of the same plate shows a series of germ tubes of varied shapes and increasing lengths before the formation of the appressoria. Long germ tubes are very seldom found (fig. 3). Very long superficial germ tubes will fail to make an appressorium and probably will never succeed in penetrating (pl. 17, fig. 5, a). In the case of *Stagonospora Curtisii*, Creager (1933) shows that the very long and several times septate superficial germ tubes form an appressorium, thus providing for good penetration. Figure 5 shows, at b, an unusual case of germination where one cell has two superficial germ tubes and the other cell, one. Figure 4 shows cases of spores germinated at both ends. The short germ tube forms a penetrating peg, the other will remain superficial. The superficial germ tubes, after having reached a certain length, may lay down cross walls.

Hasselbring (1906) sowed spores of *Gloeosporium fructigenum* in "convex" drops of water on slides kept in a moist chamber. The spores germinated differently according to their position in the drop. Those at the bot-

tom of the drop formed a short germ tube, which enlarged into an appressorium in contact with the glass. The other spores in the drops had germ tubes that floated. The same experiment was made by the writer with spores of *Diplocarpon Rosae*. No differences in germination appeared among the spores of the same drop, and no appressoria were formed.

It may be found that while about 50% of the germinated spores from drops placed on the upper side of a leaf are in a stage ready for penetration, a lower percentage will be found in the case of drops on the lower surface. It is easy to understand why some of the spores in a hanging drop, since they must develop long germ tubes in order to reach the cuticle, are in an unfavorable position for penetration. The method of penetration on the lower and the upper sides of the leaf was found to be the same.

Appressoria may vary greatly in shape as shown in figures 2 and 3. When the spore germinates from the side that touches the cuticle, the appressorium appears circular (pl. 17, fig. 1, d; pl. 18, figs. 10 and 15). These organs are not as conspicuous by their size or shape as they are by the deep staining they take where they come in contact with the cuticle, and by the rays of the dark blue stain which usually surround them (figs. 2 and 3). It is possible that these rays represent cracks made in the cuticle, which is waxy and brittle, by the firm pressure of the appressorium. The stain accumulates in these cracks and forms the blue rays visible around most of these organs. In other cases, instead of pronounced rays, a light blue area is seen immediately surrounding the appressorium (fig. 3).

Diplocarpon Rosae is like certain other fungi in that appressoria are not always formed. In a series of inoculations made in November, all of the preparations showed good stages of penetration from germ tubes which had not formed appressoria. The end of the germ tube showed a light circular spot surrounded by a darkly stained border (fig. 4). These spots which are found also in the center of appressoria (figs. 6–24) represent the so-called "place of germination" of the appressorium where the little infection peg will be sent out to penetrate the cuticle.

In stages older than nine hours, the fungus is seen to have already penetrated the cuticle. The narrow infection peg sent from the germ tube tip or from the appressorium, enlarges into a subcuticular hypha immediately after it penetrates the cuticle. There is no indication at this time of any toxic or enzymatic action of the fungus so we must assume that the cuticle is penetrated by mechanical means. Penetration never takes place through stomatal openings, although guard cells are sometimes penetrated. The subcuticular hypha is very easily distinguished by its vacuolate contents. Figure 6, in plate 17, shows an infection hypha as it is found about

thirteen hours after inoculation.[2] Text figure 1, A and B, shows the ways in which the penetrating peg enlarges into the infection hypha.

The tapered end of the infection hypha continues to grow a little longer until it reaches the wall between two adjacent cells where it pushes down into the middle lamella (pl. 17, fig. 7). At this stage each infection hypha has laid down a cross wall, a short distance from the penetrating peg (pl. 17, figs. 7–9; pl. 18, figs. 10–17 and 19; pl. 19, figs. 20–24). This wall is very conspicuous because, in most cases when the cuticle is not injured, the subcuticular hypha is stained much darker in that portion near the appressorium. The stain passes down through the infection peg but it can not pass through this impervious cross wall. The importance of this provision will be noted later on.

A careful examination of these infection hyphae shows that while their beginnings can be seen almost in the same focus as the spore, the end portions appear less clear at that focus and in order to follow them distinctly one has to focus lower and lower into the cell. This indicates that while the first portion of the hypha might be strictly subcuticular, the rest of it travels, sooner or later, through the path of least resistance across the cuticularized layers until it reaches the inner layer of the outer wall of the epidermal cell (text figure 1, D and E). Sometimes, the infection hypha, penetrating the two adjacent vertical walls, produces a swelling at the point of entry (pl. 17, fig. 9).

Figure 8 shows a very common case where the hypha does not grow completely across the cell to reach the adjacent vertical walls, but tapering out into a narrow end penetrates directly down through the outer wall of the cell. Some of the infection hyphae may assume peculiar shapes (pl. 17, fig. 6; pl. 18, fig. 10, a). At b, in figure 10, is shown a hypha which apparently has branched immediately after penetration. Another peculiar case is given in figure 20, plate 19, where a very long infection hypha has passed across two adjacent walls. In many cases, instead of being straight, (pl. 17, figs. 7, 9; pl. 18, figs. 12–14, 16–17 and 19), the infection hypha has a wavy outline in passing through the upper wall (pl. 17, fig. 8; pl. 18, figs. 11 and 15). A few cases were found, where, because of the position of the spore, penetration took place through the cuticle of the wall between two adjacent cells so that the infection hypha could be detected in this wall only by two shining blue spots (pl. 18, fig. 18). The penetration of the

[2] It is necessary to point out here that the hour at which the same stages can be found, varies with conditions of infection. As might be expected many different stages can be seen on the same slide.

infection hypha through the subcuticular layers is believed to take place with the aid of an enzymatic action of the fungus, possibly accompanied by a mechanical process. This point will be discussed in another place.

Fifteen hours after inoculation under favorable conditions, the fungus is able to form a haustorium. The spore (fig. 11) has germinated and has made an appressorium immediately; the peg has penetrated the cuticle and has enlarged into an infection hypha. The hypha has laid down a cross wall. Although at the beginning it is subcuticular, it reaches deeper layers of the upper wall towards the end where it turns down, pushes in between two adjacent vertical walls and, gradually tapering into a fine thread, penetrates the wall ending directly in a haustorium (text fig. 1, D). The young haustorium shown in figure 11, is surrounded by a jelly-like substance which has the aspect of a secretion product. The haustorium appears in the preparations as a fine blue thread all along the distance where it is surrounded by this substance. Its tip end becomes enlarged only after having emerged from this deposit. The swollen wall of the host shown in the same figure remains distinct from the substance deposited around the haustorium. The deposited substance does not take any stain; it is only the haustorium that stains blue, just like the rest of the fungus. Another fifteen-hour stage (fig. 12) shows the gelatinous substance around the haustorium having a more definite form, being now made up of two parts. The haustorium is enlarged into a knob at the end. In this particular case the spore had been washed off in preparing the mount, and only the appressorium with the "germinating spot" is seen at a. A more advanced stage of the development of a "fifteen-hour" haustorium is shown in figure 13, where the knobbed end is much more developed. In this case the wall around the haustorium, and also some distance away from it in the other cell, seemed to be very much swollen and softened and showed a layered structure around the place of penetration. These cases of such exaggerated swellings are very rare. On the same fifteen-hour preparation one can find stages showing completely formed haustoria (fig. 14). The precipitated substance has taken the form of a collar or cup made up of two parts. The haustorium, as soon as it escaped from it, spread out and elongated. About the same situation is shown in figure 17, with the difference that the collar is not divided into two regions.

The fungus can also form a haustorium in fifteen hours by sending down an infection hypha which will penetrate into the lumen of the epidermal cell directly from the outer wall (pl. 18, fig. 15). If the haustorium is vertically oriented that part which comes first into vision, focusing from the top, is the basal part of the collar which appears circular. Focusing lower down, the rest of the haustorium comes into view. These cases

are the best for studying the thinning out of the tapered end of the infection hypha into the thread which penetrates the wall and forms the haustorium (text fig. 1, D and E). In these cases also the haustorium enlarges into a knob-like end as soon as it escapes from the collar-shaped deposit. Figure 16 shows on the lower side of the leaf a haustorium sent in from the outer wall. The haustorium here is more fully developed.

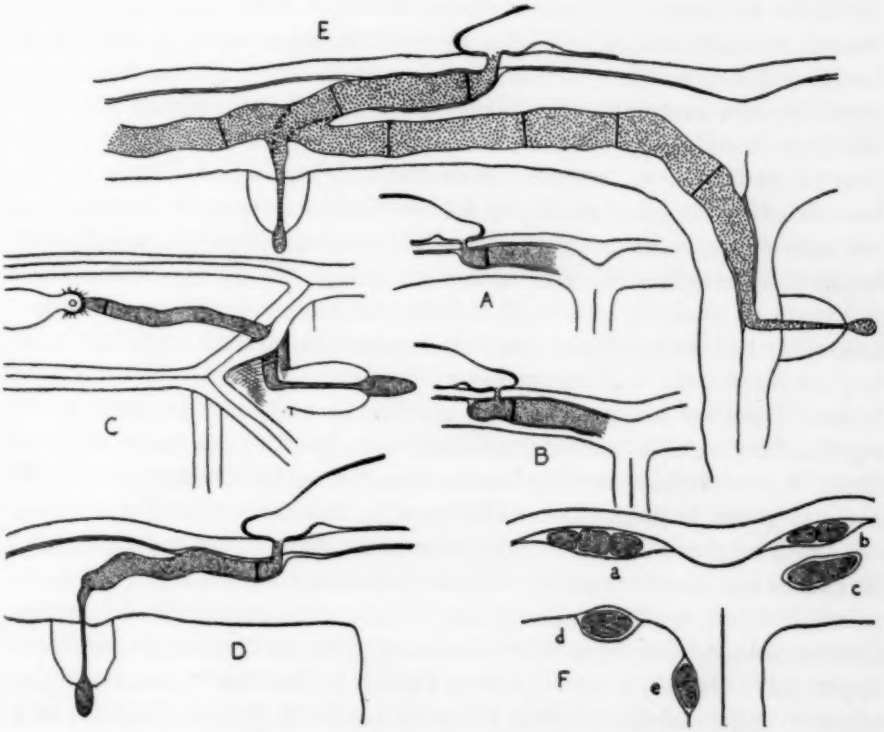

Fig. 1. A–F. Diagrams of various early stages in penetration and invasion of rose leaf by *Diplocarpon Rosae*. Exact proportions disregarded for the sake of clearness. For further explanation see text.

A more advanced stage of invasion is shown in figure 19, plate 18. This is about 29 hours old. The infection hypha has penetrated through the middle lamella and has formed a haustorium at a. Focusing lower down in the other cell a second haustorium comes into view, formed from the same center of infection by the intercellular hyphae which had grown and spread during this time. On the other side of the penetration point the progress of the intercellular hyphae could be observed by two blue spots in the wall. The connection between them was sometimes difficult to make

out because of their undulatory growth following the wall in which they developed. It is interesting to notice that the second haustorium comes from a new portion of the initial intercellular hypha. Usually not more than two or three haustoria are formed by the first pentrating hypha, and these are generally in different cells.

As a further step in invasion, additional hyphae begin to develop on the second day after inoculation. The part of the infection hypha which lies in the subcuticular layers starts to form new branches directly above the place where it has formed a haustorium (text figure 1, E). These branches travel in the upper wall and serve to spread the infection by providing new sources for the development of haustoria. Figure 20 shows the long infection hypha which has passed over the first cell and has penetrated the outer wall of the second cell into which it has sent a haustorium.[3] The infection hypha had swollen just above the haustorium and sent out branches on both sides. The original shape of the infecting hypha before swelling is indicated by dotted lines. Similar cases are shown, in figures 21 and 22, except that being a little more advanced, more branching has occurred and the new hyphae are already septate. These hyphae have quite a different aspect from that of the initial infection hypha. They are wider, their cells are shorter and their borders are irregular. Having rich contents and large vacuoles they are easily seen. In figure 21, one of these new hyphae has already reached two adjacent walls and has grown through the middle lamella. The same type of branching can occur where infection hyphae penetrate two adjacent walls (fig. 22). In a more advanced stage (fig. 23), the branching has become quite complicated. One new haustorium is formed at a, and at b and c two other hyphae are ready to form other haustoria. The swelling of the infection hypha takes place here on one side so that its initial form is preserved. An advanced phase of this stage is shown in figure 24. The haustorium at a is the one formed by the initial infection hypha; haustoria b and c were formed from branches developed from this hypha at a point just above the haustorium a. A very unusual situation is shown in the same figure at d. Here the penetrating hypha sent out a branch on the other side of the spore some time after penetration.

None of the stages described above has heretofore been given any attention in studies on the development of *Diplocarpon Rosae*. Not until about 48 hours after inoculation do the parallel strands, which others believe to be the only kind of hyphae representing the vegetative growth of

[3] For convenience, the haustoria are represented in some of the drawings only by a circle which is the base of the collar. The little circle in the middle represents an optical view of the haustorial thread.

the fungus, start to develop. Some of the branches of the secondary hyphae that we have just described for stages between 29 and 48 hours do not form haustoria, but produce hand-shaped ends (pl. 19, fig. 21, a, b; fig. 23, d; fig. 26, a). In the case shown in figure 25, the first stages of penetration could not be clearly seen. As the stages become more complicated it is difficult to find the starting point of the infection. It is quite certain, however, that the haustorium at *a* is due to the initial infection. The branched hyphae above the haustorium do not send out new haustoria but both are about to give rise to parallel hyphae. At b(fig. 25), the little side branch could grow and produce another hand-shaped formation. Sometimes, as seen at c, the finger-like projections have already laid down cross walls.

Figure 26 shows how the first cells of parallel hyphae are formed. At first they are narrow and lay down walls at short and somewhat unequal distances. In preparations stained with the triple stain, the cells are seen to be uninucleate. At the points where branching takes place, they are richer in content (pl. 20, fig. 28, a). A portion where active branching has occurred, starting from a strand of three hyphae, is shown in figure 27, plate 20. Each hypha after a certain distance of parallel growth may branch in turn and add a new hypha to the synema. If the branching continues, one can very often observe strands composed of as many as seven or more hyphae (pl. 20, fig. 32). In this case they are more irregular in shape, and sometimes leave larger spaces than usual between them. A case where two hyphae from a strand have joined another one which had already four parallel hyphae is shown in figure 28. Ducomet (1907) has given detailed illustrations of the formation of these strands as well as an adequate description and it is not necessary to discuss this point further here.

A short distance from the place where the first "parallel hypha system" originates, haustoria of a third series are sent down through the upper wall into the epidermal cells below. Their formation is the same as that described for the very first haustoria which are found at the fifteen-hour stages (pl. 18, figs. 15 and 16). Those formed from the parallel hyphae differ somewhat in shape and size (pl. 20, figs. 28, 29 and 31). In figure 28 is shown a single large haustorium. The vertically oriented haustoria are indicated only by circles. The subcuticular parallel hyphae usually do not send haustoria into cells already occupied by these organs, but, instead, into other free cells. Figure 29 shows at a, b, c and d haustoria formed from earlier stages of infection. All of the other haustoria originate from the parallel hyphae, some of them being indicated merely by circles. Two haustoria were never found coming from the same hyphal cell in a synema. Stages like those represented in figures 26 to 29, are about three to five days old.

About the sixth or seventh day after inoculation, new complications arise. The portion of the parallel hypha directly above the place where a haustorium had been formed starts to branch in the same manner as described for the earlier stages represented in figures 20 to 24, plate 19. This branch grows in the subcuticular layers of the outer wall pushing out laterally from the parent parallel hypha (fig. 30, a, b, c). Such hyphae serve as an additional means for spreading infection and for the formation of haustoria. These "scouts" are much wider than any of the hyphal branches of this fungus described before. They have varied shapes, irregular borders and a foamy, cytoplasmic content which, when stained, makes the many large vacuoles more evident. They are clearly the same hyphae that Dodge (1931) refers to as "haustorium mother-cells." They may be either one- or two-celled. These hyphae reach two adjacent walls (fig. 30, c) and immediately form a haustorium, usually in a cell which is free from infection (figs. 31 and 32). Figure 31 shows at a one of these scout hyphae that is ready to send its tapered end directly through the upper wall to form a haustorium. During this stage of infection five or even more haustoria can be found in a single cell (fig. 31).

In order to give a better understanding of the position of the different kinds of hyphae that develop in the epidermal walls a schematic drawing is given at F, in text figure 1. The parallel hyphae at a and b are usually just beneath the cuticle. This is seen in cross sections of imbedded material and can be observed from the top of the leaf, where they appear almost in the same focus as the spores and above all the other hyphae. When they first start to form as shown in figure 26, plate 19, careful focusing indicates that they occupy a lower level in the wall but, soon after, come up directly beneath the cuticle. In cases like those shown in figure 28, plate 20, where one synema passes underneath another, the hyphae composing the lower one are either making their way by pressing down in their growth through the cuticularized layers, or they might be growing right in these layers. The hyphae come up to a higher level again as soon as the "crossing place" has been passed. All the other hyphae borne on the different systems of branching found in the early stages, including the "scouts," occupy a lower position in the layers of the wall as seen in the text figure at F, c, d and e. These observations made from sectioned material are confirmed by careful focusing from the cuticle down into the wall.

MORPHOLOGY OF HAUSTORIA AND RELATION BETWEEN HOST AND PARASITE

An attempt to review all the literature on haustoria seems unnecessary here, as Rice's (1927) detailed discussion and bibliography cover this subject very thoroughly. The writer is concerned here with noting only some

of the earlier papers regarding haustoria, which have been consulted in order to better understand the morphology of these organs as found in *Diplocarpon Rosae*. It seems necessary to discuss here in particular the formation and the meaning of the haustorium "sheath." The term sheath, although attributed by all the workers mostly to the result of a reaction of the host cell against the invading hypha, is not used in the same sense by different authors. Its appearance, time and place of formation differ widely in the various accounts.

When the wall of the haustorium remains thin during its development, some authors report the absence of a sheath. Sappin-Trouffy (1892, 1896) reports thin-walled haustoria in the cases of different species of *Uromyces* and *Puccinia*. Pole Evans (1907) reports no sheath for the young haustoria of the cereal rusts and Dastur (1913) for *Phytophthora parasitica*, also remarks on its absence.

De Bary (1870) in the study of *Erysiphe* says that the sheath-like layer covering the haustorium belongs to the host cell. He considers that this stage represents a haustorium which was checked in its growth. Sappin-Trouffy (1896) reports that *Uromyces Betae* forms haustoria with a much thicker membrane than that of the intercellular hyphae. Mangin (1895) studying the Peronosporaceae states that in all of the species observed the haustoria are never free in the host cells. They are protected by a sheath which does not adhere to these organs. The sheath, according to him, is made up entirely of callose or has some cellulose at its base near the cell wall. It is formed all around the haustorium at the same time that it elongates and thickens in the cell cavity. This formation makes the haustorium appear much larger and with rough irregular borders. Istvánffi and Palinkás (1913) make the same observations in the case of *Plasmopara viticola*.

Ducomet (1907) considers the sheath of *Cuticularia Ilicis* on *Ilex* as a region of digestion, a clear zone with a dark fringed border. Reed and Crabill (1915) report for haustoria of *Gymnosporangium Juniperi-Virginianae* on cedar, an envelope or capsule which stains differently than the fungus.

Smith (1900), in the case of several Erysiphaceae studied, is more explicit in his description of the origin of the sheath. As soon as the haustorium starts to penetrate the cell, an ingrowth from the cell wall begins to form in the shape of a collar. He considers this as an addition of new material to the wall which takes place at the same time with the advancement of the haustorium in the cell. He suggests that the production of cellulose around the region of penetration might be due to some chemical substance excreted by the fungus which excites the cell protoplast to an unusual activ-

ity. As soon as the haustorium has escaped from the collar-like ingrowth, according to Smith, a part of this deposited substance begins to disintegrate. Its products of disintegration, by accumulating between the haustorium wall and the host plasma membrane, make the sheath appear much more definite at this stage. Higgins (1914) describes for *Cylindrosporium* on stone fruits, sheathed haustoria which he thinks resemble those described by Smith.

In many cases sheathed haustoria were reported only for older stages of development of these organs. Tischler (1912), for haustoria of *Uromyces Pisi* on *Euphorbia cyparissias* and of *Albugo candida* on *Capsella bursa pastoris*, Colley (1918) in the case of *Cronartium ribicola* on *Pinus Strobus*, and Moss (1926) for certain species in the *Pucciniastreae*, report sheath formations around mature, old and even dead haustoria. Rice (1927) reports two kinds of thickenings for haustoria of different rusts. She finds some cases where a ring-like thickening is formed some place about the stalk and which she attributes either to a differentiation of the outer surface of the haustorial wall or to a deposit from the host cytoplasm. Some stages in the corn rust show sheath-like thickenings either around the stalk itself or also around the lobes of the haustorium. The formation of this sheath seems to take place almost always around the old haustoria. Rice tries to explain the formation of the sheath as a result of less vigor on the part of the fungus. The sheath is formed very often when the haustorium is forced to enter thick-walled cells.

In the case of some parasitic fungi sheaths in the form of cellulose layers deposited along the hyphae which cross through the cells of their host are also reported. Wolff (1873) in the case of *Urocystis occulta*, Butler (1907) in the case of *Sclerospora graminicola* on cereals, and of *Ustilago Zeae* (1918) on corn, report that hyphae which pass through the cells are clothed with a distinct cellulose sheath, which they consider as deposited by the host cells in a defensive attempt to check the fungus in its growth. Leitgeb (1881) figures them also for the penetrating filaments of *Completoria complens* in fern prothallia. His figures show that he was dealing with true haustoria.

We shall consider now the young stages in the development of the haustoria of *Diplocarpon Rosae* and attempt to identify some of their structures with those described above for other fungi. The first sign of formation of a haustorium is in many cases a swelling of the wall of one of the two adjacent cells, at the place of a penetrating hypha (pl. 19, fig. 25, d, e). The hypha pushes in between the two walls by way of the middle lamella, as can be seen in some of the preparations which show this layer very distinctly. Haustorium mother cells which are quite frequent in the

different rusts, as reported by Pole Evans (1907), Reed and Crabill (1915), Allen (1923a, 1923b) and Dodge (1918, 1922, 1934), are not present in this fungus. The ends of the hyphae thin out into thread-like formations which penetrate the wall, without the formation of a preliminary end cell. At this stage, the fungus sends out a very thin filament which pierces the wall and develops into a haustorium inside the cell.

Very early stages are the only ones which indicate that the plasma membrane is merely invaginated rather than pierced by the invading fungus (pl. 19, fig. 21, c, d; pl. 20, fig. 30, d, e). They also show the haustorium as a very fine thread which has already enlarged at the tip. At first the thread-like part is surrounded by some deposited substance as noted previously, and which is slightly granular and irregularly shaped (fig. 21, d and e). A more advanced stage shows this substance taking the shape of a collar (figs. 22, a, b; fig. 26, b, c). In some cases very little of this substance is deposited (fig. 21, e) so that the haustorium swells up immediately; more frequently the deposit is present in larger quantities and prevents the haustorium from enlarging; only at the tip does the fungus thread escape and swells up as seen in figures 22 and 26. This substance assumes a more definite shape in older stages and can be made out as composed of two parts (pl. 20, fig. 28, b, and fig. 31, b) or as only one part (fig. 28, c). We consider that these formations found around the haustoria of *Diplocarpon Rosae* resemble very much the cellulose collar deposited by the wall of the cell around the haustoria described by Smith (1900). The collar does not increase in size much after it is once formed. It does not seem to disintegrate as reported by Smith for the Erysiphaceae. Sheaths which entirely surround the haustoria have not been observed by the writer.

After the collar has developed completely, the haustorium enlarges and elongates. No lobed or branched haustoria are found in the case of *Diplocarpon Rosae*. They are usually uninucleate and have finely granular contents which stain darkly with cotton blue. A great number of haustoria are sent into the epidermis. They can be found also in the palisade cells in later stages (Dodge, 1931) but were not observed in the cells of the spongy parenchyma.

The haustorial complex formed in earlier stages attains rather large sizes and varied shapes. Including the collar it may be either very long so that it nearly reaches the opposite wall of the cell (pl. 18, fig. 19), or the collar may develop more in width, the haustorium itself thus remaining short (pl. 19, fig. 23, a). In any case, the collar accompanies the haustorium to about half of its length so that not very much direct contact between the fungus and the cytoplasm of the host cell is possible. Haustoria which are

formed in older stages of development, especially those sent out by the "scouts," are very often somewhat smaller. The collar also is less prominent in these cases (pl. 20, figs. 31, 32).

Numerous cases were found where the haustorium was in contact with the nucleus but just as many were noted in which the nucleus occupied a position near the cell wall with no connection at all with the haustorium. Even when in contact with the haustorium, no important deformation was observed as described for other fungi.[4]

Interesting studies made by Dufrénoy (1928, 1929, 1930, 1933) aim to bring out the different changes that take place in the cytoplasm of the host under the influence of parasites, fungi, bacteria and viruses. The most important changes observed in vital stained tissues is concerned with the vacuolate apparatus. He cites different examples of influence of the parasite on the host, such as *Colletotrichum Gloeosporiodes* on *Citrus*, *Peronospora Schleidenii* on onion, *Phytophthora infestans* on potato, *Endothia parasitica* on chestnut, *Uromyces Caladii* on *Arisaema triphyllum*, and *Helminthosporium* on *Hordeum*, and about the same general phenomena are reported. In the case of a cell parasitized either by a hypha or by a haustorium, the single large vacuole, which was present in the cell before the invasion, is shown to break up into small ones. The cell finally appears plasmolyzed. The fragmentation of the vacuome seems to correspond to an exaggeration of the proteolitic processes which is expressed morphologically by an increase in the surface of contact. This fragmentation is nothing but an indication of a reversible evolution from a cell, which was almost at rest, to a period of active metabolism. In the case of some fungi, this fragmentation appears also in the neighboring, non-parasitized cells.

Rice (1934) points out that her detailed studies made on relations of rusts to their hosts have not revealed any changes in the host cell protoplasm around the haustorium. With special reference to *Uromyces Caladii*, she states that no intracellular hyphae were observed for this fungus as represented by Dufrénoy. She also states that *Uromyces Caladii*, like other rust fungi, being a highly adapted parasite makes but little appreciable alteration in the physical condition of the cytoplasm and that no plasmolysis of the host cytoplasm can be observed.

Our observations on this subject in the case of *Diplocarpon Rosae* reveal in a few cases a system of small vacuoles which are gathered around the haustorium in different stages of its development (pl. 20, fig. 30, d, e, f). A much more careful study is needed using vital stains which might bring out more evidence on their presence and origin.

A word regarding the browning of the cells attacked by *Diplocarpon*

[4] See Rice (1934) for literature on this subject.

Rosae may not be out of place here. Our detailed studies on penetration could not establish a definite time when the browning of the cells occurs, as many variations were found in this condition. The brown spot on the infected part of the leaf can be seen in the more favorable cases as early as three days after inoculation. By boiling the leaf in alcohol, it could, however, be seen in preparations of earlier stages. The microscopical observations sometimes indicate an incipient browning as early as twelve hours after inoculation before the haustorium is formed. Many cases show that the browning of the cells may start immediately after the formation of the first haustorium. Sometimes it is delayed even after the formation of the parallel hyphae. Cells that are invaded by a larger number of haustoria become brown very quickly. In later stages of penetration the infected cells are dark brown and their old and deformed haustoria can scarcely be seen. Wolf (1912b, 1913) attributes the color of the infected spot merely to the browning of the substances in the host cells. We are more inclined to agree with Ducomet (1907) that the parallel hyphae which are also seen to be colored in older stages, probably contribute to the coloration of the spot.

CONTROL MEASURES AS CORRELATED WITH DATA ON INFECTION

Of the "wet sprays" Green (1931) and Parsons and Massey (1933) state that Bordeaux Mixture is the best, although White (1930b) reports that copper sprays seem to produce a dwarfing of the plant in addition to the dwarfing due to the black spot that persisted.

Massey and Westcott (1928), Dodge (1932), and Massey and Parsons (1931) have emphasized the importance of very careful spraying or dusting just before rains. During rainy periods it may be necessary to dust or spray two or three times a week.

Our inoculation experiments have shown that rainy periods with not very high temperature are most favorable for infection. We have shown also that in about nine hours of favorable conditions the spore germinates and that in about thirteen hours after inoculation the fungus has penetrated the cuticle and formed the infection hypha. From this time on, it seems to be able to protect itself against any unfavorable climatic conditions because of an impervious transverse wall which is laid down by the penetrating hypha, just beyond the penetration peg, in the subcuticular layers. By applying fungicides at short intervals of time before precipitation and during rainy periods, the spores can be kept from germinating or from developing infection. We can now better understand why Dodge (1929, 1932) and Parsons and Massey (1933) who have been carrying on experiments on black spot control for several years, agree that the fine

sulphur dusts with arsenate of lead added, are very satisfactory especially if green-sulphur dust, which does not discolor the foliage, is used. White (1932) finds that sulphur sprays adhere well and thus give good control.

DISCUSSION AND CONCLUSIONS

Our experiments have shown that the time required for the spots to appear can be reduced to three days; that infection is very little affected by the absence of light, because the fungus is capable of living saprophytically. The abnormal condition of the plant placed in the dark does not suppress or retard to any great extent the development of the fungus. While Fromme (1913) has shown in the case of *Puccinia coronifera* that total light exclusion checks the growth of the fungus, our results agree more with those of Lauritzen (1919), who concluded that light is not a limiting factor if experiments are not conducted at the border of favorable temperatures and humidity.

The appressoria of *Diplocarpon Rosae* are represented only by the swollen part of the enlarged germ tube, which stains darker at the point where it touches the cuticle. They are not definite organs as described by De Bary (1886) for *Sclerotinia* where they can appear in tufts, or as brown thick-walled spore-like bodies as described by Hasselbring (1906) for *Gloeosporium fructigenum*. Appressorium formation is recorded by Allen (1923a), as taking place on the day following the inoculation. Hasselbring (1906) reports their formation from twelve to eighteen hours after inoculation, while in *Diplocarpon Rosae* they appear as early as the ninth hour.

Nordhausen (1899) and Büsgen (1893) for *B. cinerea*, Dastur (1921) for infection with sporidia of *Tilletia Tritici* and Weber (1922) in the case of the corn rust, report that penetration occurred more frequently near the boundaries of epidermal cells than over the lumen of the cells. They consider that these places are more convenient for the exosmosis, from the content of the cell, of a chemotropical substance which would favor penetration. A second reason is that the easier way for a penetrating hypha to enter is through the middle lamella. Our observations on penetration by the black spot fungus show very little preference one way or the other.

The rose cuticle seems to be pierced wholly by mechanical means. The sudden enlargement of the infection peg into an infection hypha as soon as the cuticularized layers are reached, indicates that further penetration must take place in a different way in this region. We could not expect such a wide hypha to push its way through merely by mechanical action, although it is certainly flattened out considerably, which would suggest that it advances under pressure from the elastic cuticle. The lenticular pockets seen in the epidermis wall in cross sections of embedded material,

are usually greater in diameter than the hyphae which have formed them (text fig. 1 F, c, d, e). This indicates a chemical action of the fungus in making its way through the wall into which the enzyme produced has diffused a little beyond the boundary wall of the hypha. Ducomet (1907) claims that the regions around the penetration of the hyphae in the layers beneath the cuticle are the only ones in the host wall which take the cotton blue stain indicating therefore a change in the composition of the wall. The blackish cement that unites the parallel hyphae shows, according to him, traces of cellulose when colored with Congo red. He believes that the membrane is digested and that the intercalary substance is cellulose, chemically changed. We feel that the swellings of the wall so obvious in many cases at the places where haustoria will be sent through, indicate that an enzymatic action brings about the softening of the wall. Much swelling of the host wall is reported by Ward(1888) in the case of *Botrytis Tulipae* and by Blackman (1916) for *Botrytis cinerea*.

In the case of rusts especially, and of other fungi that form very fine haustorial threads, it is not improbable that penetration should take place merely by mechanical means. For *Diplocarpon Rosae* this might be the method by which a haustorium is sent into a cell from a hypha which is already intercellular (text fig. 1, C, E).

The first haustorium can be formed as early as fifteen hours after inoculation. Pole Evans (1907) reports that haustoria appear the third day from the uredo-mycelia of different rusts, and Higgins (1914) for *Cylindrosporium* on stone fruits observed them only after the fifth day. Twelve hours of favorable conditions are sufficient to insure infection and development of spots. The impervious cross-wall laid down by the fungus very soon after penetration of the cuticle may be a means of protecting the parasite against drying out and insuring its invasion regardless of later weather conditions.

The different types of hyphae developed by the fungus in the stages described are always intercellular (text fig. 1, F). The "intracellular" hyphae figured by Ducomet (1907) and mentioned by Wolf (1913) were evidently the "scouts" which are seen sometimes growing in the upper wall of the epidermis above the lumen of a cell, (pl. 20, figs. 30, c, and 32, a, b, c), giving the appearance as if they were intracellular. Cross sections of leaves show that scout hyphae are sometimes found in the lower layers of the outer wall, but even then they are separated from the lumen of the cell by one or two very thin layers (text fig. 1, F, at c and d). One finds intracellular hyphae in *Diplocarpon Rosae* only as a very late saprophytic growth of the fungus, when the stroma starts to form. As we know, this occurs late in the autumn when the leaves have fallen.

The collar formed around the base of the haustorium seems to be, in its time and manner of formation, very much like the collar described by Smith (1900) for the Erysiphaceae. It differs from it, however, in that its deposition never occurs in advance of the haustorium, but keeps pace with it until it is fully formed. This might indicate that in the case of *Diplocarpon Rosae* the stimulating effect of the fungus extends only a short distance in advance of the haustorium.

All other investigators who have studied the collar and sheath of the haustorium agree, as we have already noted, that the formation of these inert parts of the haustorium is a result of the reaction of the host to the penetrating fungus. The question of proving the nature of these structures by using the proper stains or perhaps polarized light, remains open. It would be interesting to find out why the collar found is so often composed of two parts. Does this indicate that the host has ceased the deposition of the substance for a time and then has started, with renewed activity?

The collar at the base of the haustoria formed at the end of scout hyphae and from the cells of the synema are smaller than are the collars of the haustoria formed earlier. This might indicate that at the time when these are formed the attack on the leaf is so advanced that the cytoplasm has lost part of that reactive power which has an effect on the deposition of collar material.

Dufrénoy (1928, 1929, 1930) in presenting his observations on the influence of different parasites upon the cytoplasm of their hosts does not note any difference, as we have already seen, between the results in the behavior of obligate parasites and of facultative ones. It seems that different effects and more serious ones must be expected in the cytoplasm of a host cell which has been penetrated by a hypha of a facultative parasite which does not form any haustoria, than the effects produced by a haustorium which has invaginated only the cytoplasm.

Brown (1915) and Blackman and Welsford (1916) report that cells of bean pods inoculated with *Botrytis* are killed right after penetration of the cuticle. The same effect on the host is reported by Stevens and Hawkins (1917) for *Rhizopus*. By piercing the cells and killing the protoplast it produces the "leak" of strawberry. Hawkins and Harvey (1919) believe that *Pythium* punctures the cells of the potato tubers and then destroys their contents by secreted toxins. In the case of *Phytophthora infestans* on potato tuber it is reported by Jones, Giddings and Lutman (1912), that the host cells continue, for a short time only, to manufacture starch in the presence of the parasite; they die soon after they have been invaded. It might be also, according to these authors, that the attack is so sudden that the cell is unable to secrete the necessary diastases and is

caught with its chloroplasts full of starch.

Hartig (1882) considers as parasitic a number of Hymenomycetes which produce decomposition of the wood of living trees. In the case of *Septogloeum Hartigianum* (Hartig, 1892) on *Acer campestris* he reports that the mycelium is intercellular as well as intracellular and that haustoria are developed in the inner parenchymatous cells. On the other hand many cases are reported for strictly parasitic fungi where haustoria and cytoplasm of the host live for a long time without any sign of disturbance in the infected cell. De Bary (1887, p. 392) makes a very distinct difference between these two kinds. Ward (1902, 1905) when discussing the relations of rusts to their hosts says that they are different from those of a facultative parasite. They do not live upon the protoplasm of their host but consume only the food, and usually do not appear to injure the cells of the infected areas. Tischler (1912) in the case of *Euphorbia Cyparissias* attacked by *Uromyces Pisi* mentions that the haustorium in the host cell produces no disturbance for quite a long time. Allen (1923a, 1923b) reports the same behavior in the cells of Baart wheat which is susceptible to *Puccinia graminis Tritici* forms III and XIX. These are only a few of the many cases reported in the literature. Even among rusts, however, there are differences in the time when disturbance is first evident in the cell. Dodge (1934) reports that while cells of *Juniperus* penetrated by haustoria of *Gymnosporangium germinale* show very early disorganization, those of *Chamaecyparis* parasitized by *G. myricatum* are rejuvenated and have their life greatly prolonged.

In the case of *Diplocarpon Rosae* we have seen that browning of the cells penetrated by haustoria can occur at different times after infection. However, as yet, we do not know exactly how this browning and death of the cells occurs. Is it due merely to an accumulation of toxic substances or is it only a result of a premature drying out of the cells? A case comparable to this could be *Cylindrosporium* infecting stone fruits where Higgins (1914) reports that the death of cells is brought about by drying rather than by a toxic substance from the fungus protoplasm.

The fact that *Diplocarpon Rosae* forms haustoria in the rose leaf as early as fifteen hours after inoculation would seem to indicate that this fungus has reached a high degree of adaptation to parasitism. We know that in the case of many adapted parasites the haustoria keep on developing after their entrance and by branched, lobed or other complicated growths succeed in providing an extensive surface of contact between themselves and the host cytoplasm thereby increasing osmotic exchanges.

Haustoria of *Diplocarpon Rosae* do not develop much in surface before reaching maturity. We have seen also that a great number of them are

sent into the epidermis which has a poor supply of food. We are therefore inclined to consider that the fungus is capable of taking food also with the aid of the intercellular mycelium. The enzymatic action on host cellulose could break down this substance to a form which might be absorbable by the fungus. On the other hand, on the theory of structure of the cell wall advanced by Hansteen-Cranner (1922), there is a possibility that the intercellular hyphae might draw food by exosmosis from the cytoplasm. The same way of feeding must take place in the case of *Exoascus deformans* which according to Wallace and Whetzel (1910) develops subcuticularly and intercellularly but does not form haustoria. Considering these various views, one can say that *Diplocarpon Rosae* seems to be a transition form which has left the lowest step of parasitism but is still far from the level of a strictly obligate parasite.

SUMMARY

Inoculations of rose leaves with *Diplocarpon Rosae* show that the optimum conditions for infection are a saturated atmosphere and a medium temperature of about 75–80°F. In this case, spots appear on the third day. Petioles and young stems can also be infected. Of the varieties used, there is not much difference in the time necessary for the spots to appear. Infection is very little influenced by the absence of light.

Under optimum conditions, the spores placed on young leaves show complete germination in about nine hours after inoculation. By this time appressoria, if they are to be developed, will usually have formed. In the interval of 48 hours after inoculation, the fungus forms three kinds of hyphae which insure its spreading in healthy tissues and its further development in the intercellular spaces by three series of haustoria which penetrate the cells. A fourth kind of hyphal growth is developed from the parallel strands beginning about the sixth day after inoculation. These scout hyphae seek new places for penetration and formation of haustoria. The impervious cross wall laid down by the infection hypha by the thirteenth hour after inoculation, may be the means by which the parasite is protected against drying out and insures its invasion regardless of the weather conditions.

Penetration of the cuticle takes place mechanically. There is some evidence that the other layers of the wall may be modified by the chemical action of an enzyme secreted by the fungus so that they are more readily penetrated. Very likely, both chemical and mechanical action are necessary. Haustoria are surrounded by a collar-shaped basal mass which seems to be composed of material deposited by the host cytoplasm at the same time that the haustorium advances into the cell. No sheath was seen to

extend completely around the haustorium as reported for haustoria of many parasitic fungi. Browning of the host cells may start during the very early stages of invasion. This browning is mostly responsible for the dark color of the leaf spot.

Diplocarpon Rosae seems to represent a transition stage of adaptation to parasitic life. It has not as yet reached the level of a high type of parasitism.

This work was undertaken at the suggestion of Dr. B. O. Dodge and has been prosecuted throughout under his guidance. It is a great pleasure for me to take this opportunity of recording my indebtedness to him for his interest and valuable assistance.

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Explanation of plates

For a correct interpretation of the drawings see page 304 and 305 in the text.

The insets in some of the figures of plates 19 and 20 showing details in early stages of haustorium formation, are explained under "insets" at the end of the explanation of plates.

Plate 16

Artificially inoculated rose plant of the variety Red Radiance. The spots appeared the third day after inoculation. The photograph was taken two weeks later.

Plate 17

Fig. 1. Early phases in the germination of spores. About nine hours from inoculation. Shield-like thickenings indicate the places of germination.

Figs. 2 and 3. More advanced phases of germination; superficial germ tubes have formed appressoria. Shield-like thickenings and the rays surrounding the appressoria plainly visible. About ten hours old.

Fig. 4. Stages of germination, about ten to twelve hours old. No appressoria were formed at the end of germ tubes in these cases. The infection peg present.

Fig. 5. At a, a long superficial germ tube; at b, unusual germination.

Fig. 6. "Infection hypha" starts to form about thirteen hours after inoculation.

Figs. 7 and 9. Infection hypha penetrating in between two adjacent walls. Note the wall laid down across the hypha a short distance from the penetrating peg.

Fig. 8. Infection hypha in a thirteen hour old preparation, penetrating the outer wall of the epidermis.

Plate 18

Fig. 10. At a, unusually wide infection hypha; at b, the infection hypha has branched soon after penetration.

Figs. 11 and 12. "Fifteen hour" stages showing young haustoria with the deposited substance surrounding them.

Fig. 13. An older stage in the formation of the haustorium. The fungus thread has enlarged into a knob after escaping the collar-shaped deposit. Note the exaggerated swelling of the host wall.

Figs. 14 and 17. Mature haustoria found in fifteen-hour preparations. In fig. 14 the collar is shown composed of two parts, and in fig. 17 of only one part.

Figs. 15 and 16. Young haustoria, of the same age as the preceding, formed from an infection hypha which has penetrated directly through the outer wall.

Fig. 18. A case of penetration where, because of the position of the spore, the infection hypha has grown down directly between the two adjacent walls.

Fig. 19. Twenty-nine hour stage. At a, the first haustorium is formed directly from the infection hypha. A second haustorium, formed a little later and from a new portion of the initial intercellular hypha, appears lower down in the other cell.

Plate 19

Figs. 20–25 show another infection stage found in preparations from 36 to about 48 hours old. Some haustoria are indicated only by circles. The initial form of the end of the infection hypha is indicated.

Fig. 20. The branching of the infection hypha right above the place where the haustorium was sent in.

Figs. 21 and 22. More advanced stages of the same type of branching. In fig. 21, one branch has penetrated between two adjacent walls. At a and b the hand-like ends are ready to form parallel hyphae.

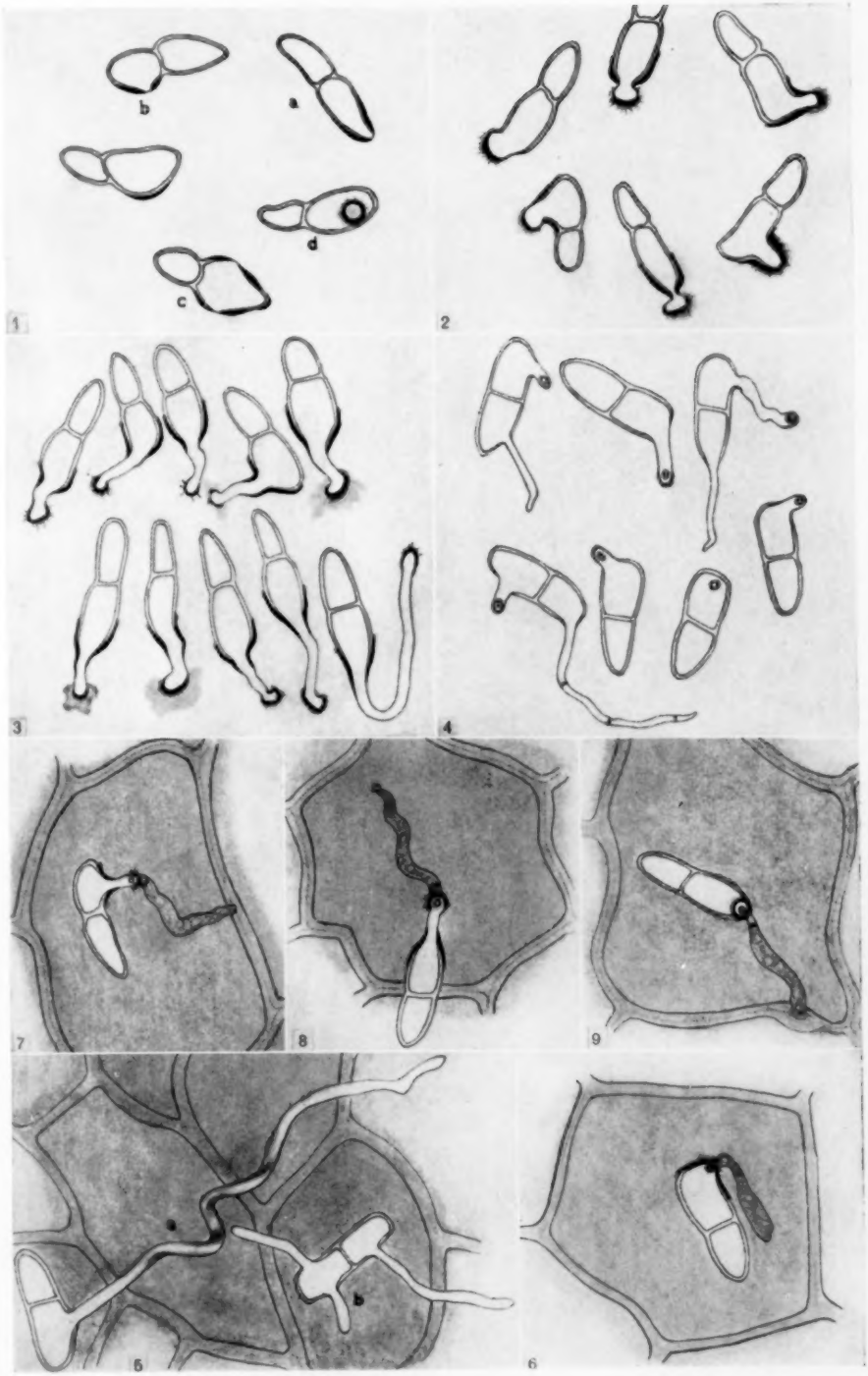
Fig. 23. Further development of the same stage. Haustorium formed at a. The branches at b and c are prepared to form haustoria also. At d, the branch will give rise to parallel hyphae.

Fig. 24. The haustorium at a was formed at the initial infection. Haustoria at b and c were formed from the second type of branches the ones developed from the infection hypha just above the place where haustorium a was sent into the cell.

Fig. 25. 48 hour stage. Haustorium at a, formed probably by the initial infection. Note the hand shaped ends of hyphae which will form the parallel strands. At b, a little side branch which could form another hand-shaped growth. The finger-like projections at c, have already laid down cross walls.

Fig. 26. First cells of the "parallel hyphae." They are uninucleate.

ARONESCU: DIPLOCARPON



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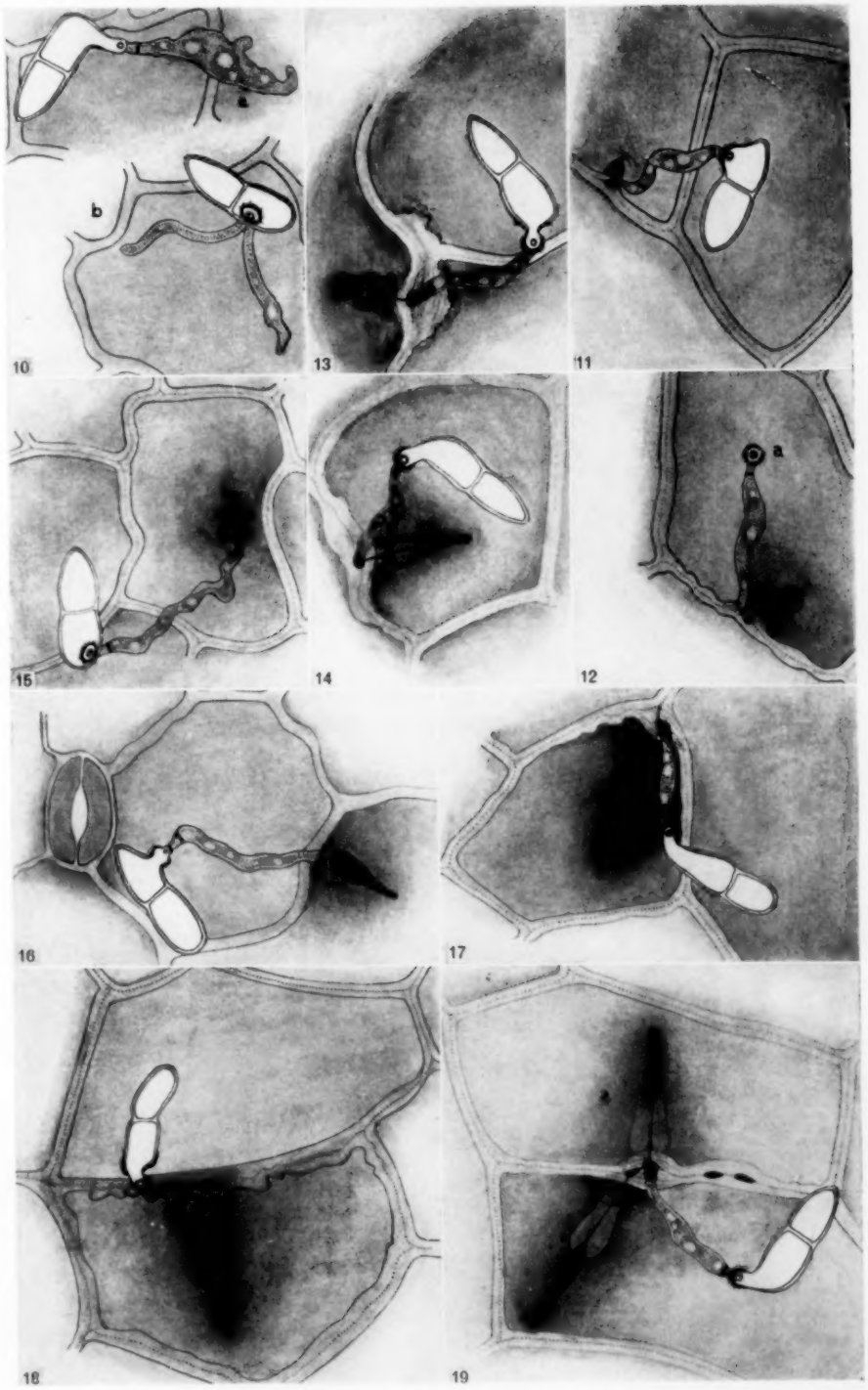
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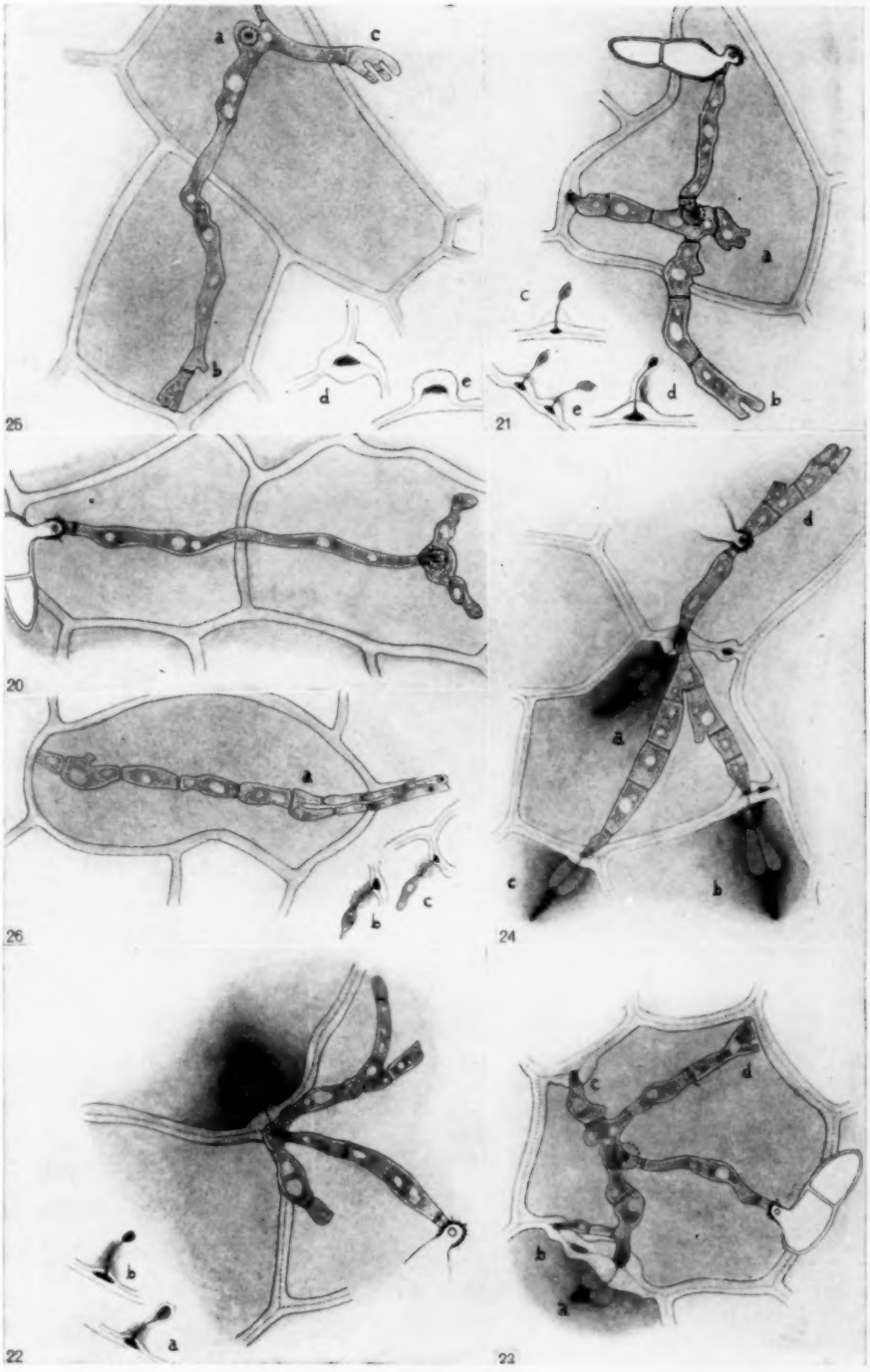
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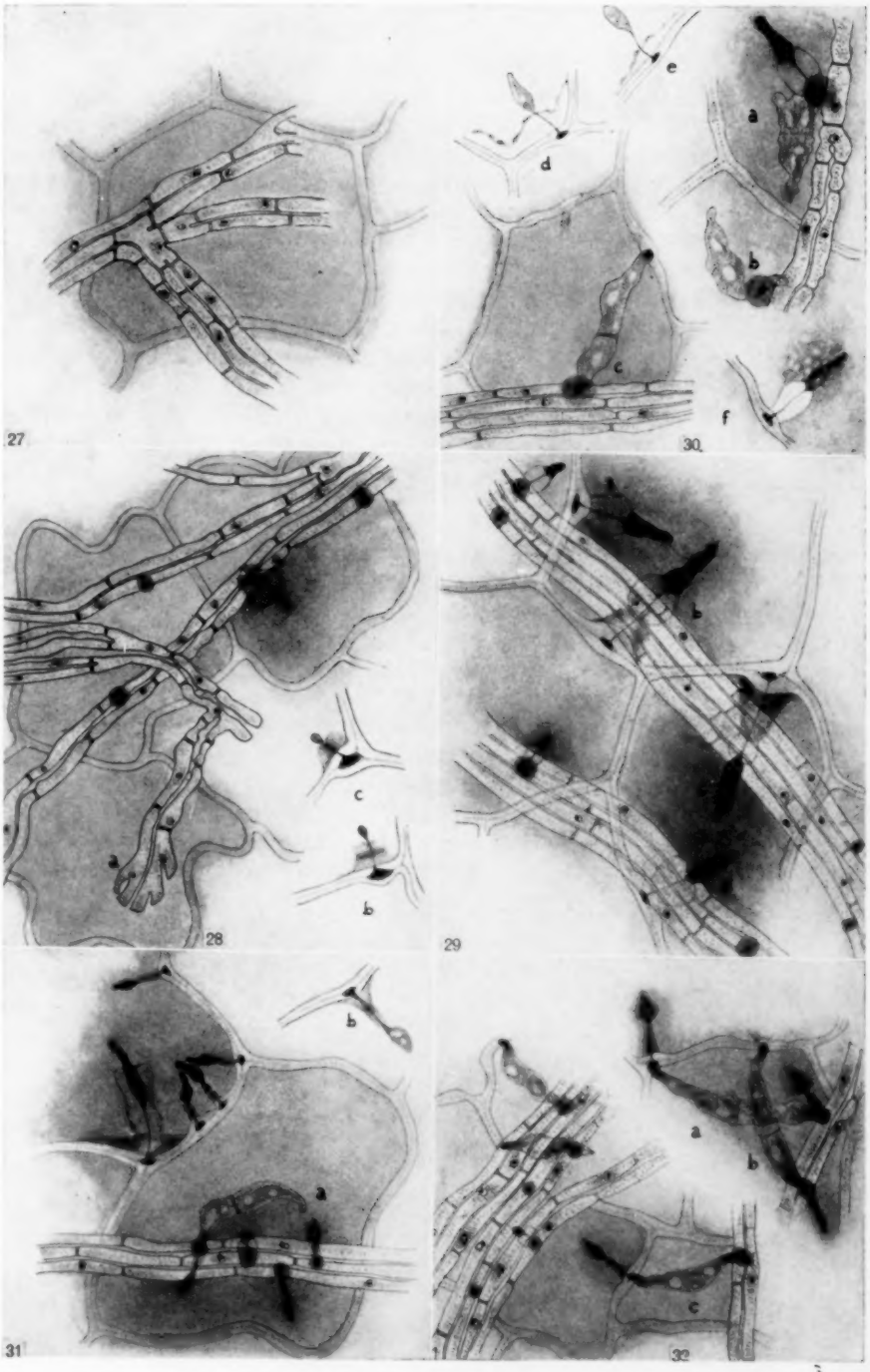

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Plate 20

Haustoria indicated in many cases only by circles.

Fig. 27. Active branching of parallel hyphae starting from a synema of three hyphae. Three days old.

Fig. 28. At a, a center where active branching has started. The figure shows different ways of association among the hyphae of different synemata. A few haustoria formed by the parallel hyphae are also shown. Three to five days old.

Fig. 29. The haustoria at a, b, c and d, were formed during earlier stages of infection. All the others originate from the parallel hyphae. Three to five days old.

Fig. 30. At a and b, formation of scout hyphae, by the branching out of the parallel hyphae, directly above the haustorium. At c the scout has penetrated between two adjacent walls. About six to seven days old.

Figs. 31 and 32 show numerous scouts which have formed the "fourth series" of haustoria. At a, b, c (fig. 32) the scouts are growing in the cuticularized layers above the lumen of the cell.

Explanation of "insets" of plates 19 and 20

Fig. 25. At d and e swelling of the wall at the place of penetration.

Fig. 21. At c and d young stages showing invagination of the plasma membrane of the host. Very little substance is deposited around the haustoria at c, d and e.

Fig. 22. At a and b, young haustoria surrounded by the deposited substance. The tips of haustoria enlarged into a knob.

Fig. 26. At b and c, the deposited substance has taken the shape of a collar. Haustoria are more enlarged at their tips.

Fig. 28. Young stages where the deposited substance is more abundant. At b, the collar-shaped deposit is composed of two parts.

Fig. 31. At b, more mature haustorium surrounded by the collar.

Fig. 30. At f, a mature haustorium. The haustorial thread has enlarged and elongated. At d, e and f are indicated some reactions of the haustorium affecting the surrounding host cytoplasm. (See text p. 316.)

INDEX TO AMERICAN BOTANICAL LITERATURE

1930–1934

The aim of this Index is to include all current botanical literature written by Americans, published in America, or based upon American material; the word America being used in the broadest sense.

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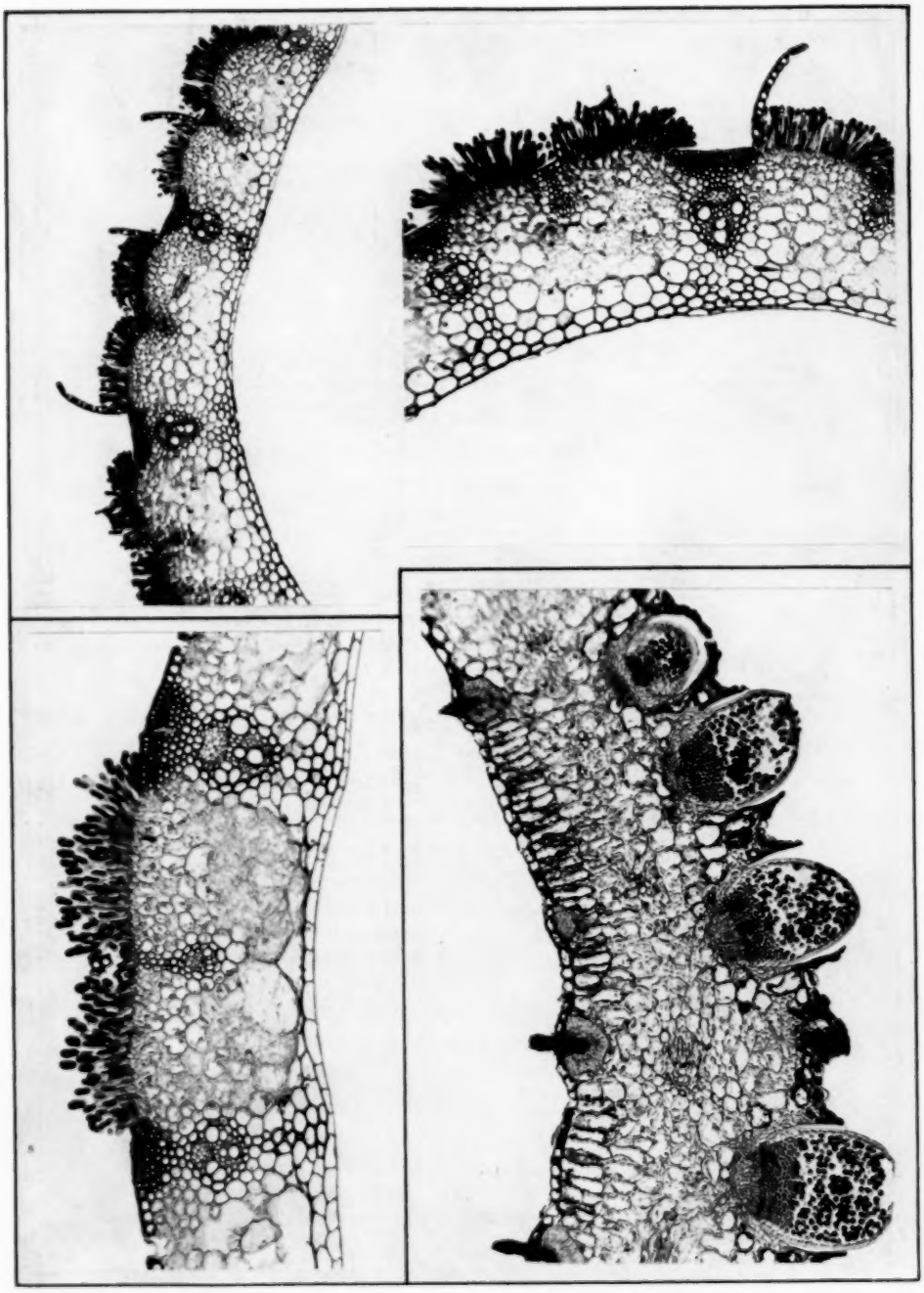

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